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(54) Title: MODIFIED DHPS GENES

(57) Abstract: The invention relates to modifications of biosynthesis key enzymes leading to essential amino acid accumulation in cells. A recombinant or isolated nucleic acid, or functional fragment thereof, encoding an enzyme dihydrodipicolinate synthase (DHPS), or functional fragment thereof, having been provided with a mutation leading to the replacement of at least one single amino acid residue with a cysteine residue.

Title: Modified DHPS genes

The invention relates to modifications of biosynthesis key enzymes leading to essential amino acid accumulation in cells.

Human and monogastric animals cannot synthesise 10 out of 20 amino acids and therefore need to obtain these essential amino acids from their diet. The essential amino acids are lysine, leucine, isoleucine, valine, phenylalanine, methionine, threonine and tryptophan. Additionally, tyrosine and cysteine, although they are not strictly essential, must be considered as such, since they are synthesised only from essential amino acids: tyrosine from phenylalanine and cysteine from methionine. The diet (herein also called food respectively feed) of man and his livestock is largely based on plant material. Among the essential amino acids needed for animal and human nutrition some amino acids, such as lysine, are often only present in relatively low concentrations in crop plants. Therefore, synthetic amino acids are usually added as supplements to grain-based and other diets, in order to increase the nutritional value of the diet. Dietary proteins are not nutritionally equivalent, which correlates with the amino acid composition of the different proteins. Feeding a diet that provides an inadequate amount of one of the essential amino acids leads to negative nitrogen balance, since the normal catabolism of proteins continues, but new synthesis for replacement is limited by the relative lack of the essential amino acid. This occurs even when the total dietary intake of protein is apparently adequate. The extent to which a dietary protein can be used for the synthesis of tissue proteins is limited by the content of the essential amino acid that is present in the least amount relative to the requirement. This is the limiting amino acid of that protein.

Many crop plants contain especially low levels of lysine. Because of this, the lysine content of plants is an agronomically important trait and various attempts have been made in the past to increase the level of lysine in plants by classical breeding, mutant selection or by genetic modification.

Regulation of amino acid biosynthesis is mainly based on feedback inhibition of the key enzymes from the pathway by the amino acid end products. Problems

encountered by increasing the essential amino acid content in plants are due to the feedback inhibition mechanism.

The essential amino acid lysine is synthesised, together with threonine and methionine, from aspartate by a complex pathway which is similar for bacteria and higher plants (Figure 1). The aspartate family pathway has been characterised in detail in *Escherichia coli* by isolation of enzymes involved in the pathway, which were later also purified from higher plants (Bryan, J.K. (1980), The Biochemistry of Plants (ed. by B.J. Miflin) Vol. 5: 403-452, Academic Press, N.Y.; Umbarger, H.E. (1978) Ann. Rev. Biochem. 47: 533-606).

The rate of synthesis of the aspartate-family amino acids is regulated primarily by a complex process of feedback inhibition of the activity of some key enzymes in the pathway by the relevant amino acid end product. The first enzymatic activity in the pathway that is common to all of the aspartate-family amino acids, aspartate kinase (AK) activity, is feedback inhibited by both lysine and threonine. In addition, lysine also inhibits the activity of the enzyme dihydrodipicolinate synthase (DHPS), the first enzyme of the pathway after the branch point that leads to the synthesis of lysine. AK catalyses the phosphorylation of aspartate to form 3-aspartyl phosphate, with the accompanying hydrolysis of ATP. Both in *E. coli* and in plants several different AK isoenzymes have been identified which are differentially inhibited either by lysine or by threonine. The product of AK activity, 3-aspartyl phosphate, is in the next enzymatic step converted to 3-aspartic semialdehyde (3-ASA), which serves as a common substrate for the synthesis of both lysine and threonine. The enzyme dihydrodipicolinate synthase (DHPS) catalyses the first reaction that is unique to lysine biosynthesis, the condensation of 3-aspartate semialdehyde with pyruvate to form 2,3-dihydrodipicolinate. In *E. coli* this enzyme is encoded by the dapA locus and appears to consist of four identical subunits as a homotetramer (Shedlarski, J.G. and Gilvarg, C. (1970) J.Biol.Chem. 245: 1362-1373). The *E. coli* dapA gene has been cloned and sequenced (Richaud, F. et al. (1986) J.Bacteriol. 166: 297-300). In plants DHPS enzyme also appears to be a single enzyme comparable to the *E.coli* enzyme. All plant DHPSs purified sofar were shown to possess activity that is strongly inhibited by lysine alone. Among the major regulatory enzymes of the aspartate family pathway in plants, DHPS is the most sensitive to feedback inhibition by its

end product (I_{50} of DHPS for lysine ranges between 10 and 50 microM). DHPS is about 10-fold more sensitive to lysine inhibition than are plant lysine-sensitive AKs (I_{50} between 100 and 700 microM) and about 100-fold more sensitive to lysine inhibition than *E. coli* DHPS (I_{50} is about 1mM) (Yugari, Y. and Gilvarg, C. (1962) Biochem.Biophys.Acta 62: 612-614; Galili, G. (1995) The Plant Cell 7: 899-906).

Several lines of evidence have indicated that in plants DHPS is the major rate-limiting enzyme for lysine synthesis. Mutants of several plant species possessing feedback-insensitive AK isozyme were found to overproduce free threonine, but exhibited only a slight increase in the level of lysine (Bright,S.W.J. et al. (1982) Nature 299: 278-279; Cattoir-Reynaerts A. et al. (1983) Biochem. Physiol. Pflanzen 178: 81-90; Dotson, S.B. et al. (1990) Planta 182: 546-552; Frankard, V. et al. (1992) Plant Physiol. 99: 1285-1293). On the other hand, a feedback-insensitive DHPS mutant tobacco plant overproduced lysine (Negrutiu, I. et al. (1984) Theor.Appl.Genet. 6: 11-20; Shaver, J.M. et al. (1996) P.N.A.S. USA 93: 1962-1966). Similar results have been reported with transgenic plants expressing feedback-insensitive DHPS or AK from *E. coli* (Glassman, K.F. (1992) Biosynthesis and Mol. Regul. of Amino Acids in Plants (ed. by B.K. Singh et al.): 217-228; Perl, A. et al. (1992) Plant Mol.Biol. 19: 815-823; Shaul, O. and Galili, G. (1992a) Plant J. 2: 203-209; Shaul, O. and Galili, G. (1992b) Plant Physiol. 100: 1157-1163). Transgenic plants that expressed the *E. coli* DHPS overproduced lysine, while those that expressed the *E. coli* AK overproduced threonine and exhibited only a slight increase in the level of lysine.

European Patent Application No. 429458 discloses a method of increasing the level of free L-lysine in a plant comprising (a) introducing a foreign gene into the cells of a plant tissue source and (b) expressing the foreign gene in the cells where a first DNA sequence of the gene encodes dihydrodipicolinate synthase (DHPS) which is resistant to feedback inhibition by endogenously produced free L-lysine. The foreign gene may comprise a second DNA sequence attached to the 5' terminus of the first DNA sequence and which encodes a chloroplast transit peptide (CTP) which localises the DHPS in the chloroplast of the cells. The plants are said to produce elevated levels of free lysine. European Patent Application No. EP 93908395 describes two isolated DNA fragments comprising a fragment encoding AK insensitive to inhibition

by lysine and a second fragment encoding DHPS which is at least 20-fold less sensitive to inhibition by lysine than plant DHPS. It is claimed that the lysine-insensitive AK causes a higher than normal threonine production and that the DHPS causes a higher than normal lysine production in transformed plants.

The same results were found when feedback-insensitive bacterial DHPS and AK enzymes encoded by the *Corynebacterium* dapA gene and a mutant *E. coli* lysC gene, respectively, were expressed together in transgenic canola and soybean seeds. Several hundred-fold increases in free lysine in transgenic seed was observed, whereas the accumulation of excess threonine that was seen in transgenic seed expressing feedback-insensitive AK alone was prevented by the co-expression of DHPS (Falco,S.C. et al. (1995), Bio/Technology 13: 577-582). U.S. Patent Application No. 5773691 relates to four chimeric genes, a first encoding a bacterial lysine-insensitive aspartate kinase (AK), which is operably linked to a plant chloroplast transit sequence, a second encoding a bacterial lysine-insensitive dihydropicolinate synthase (DHPS), which is operably linked to a plant chloroplast transit sequence, a third encoding a lysine-rich protein, and a fourth encoding a plant lysine ketoglutarate reductase, all operably linked to plant specific regulatory sequences. The seeds of the transgenic plants are said to accumulate lysine or threonine to higher levels than untransformed plants.

The invention provides a recombinant or isolated nucleic acid, or functional fragment thereof, encoding an enzyme dihydrodipicolinate synthase (DHPS), or functional fragment thereof, having been provided with a mutation leading to the replacement of at least one single amino acid residue with a cysteine residue. The presence of an additional cysteine residue for example may result in the formation of alternative or additional sulphur bridges. As a result the overall structure and/or flexibilty of the enzyme will be affected, leading in the preferred cases to the loss of lysine feedback inhibition. In the present application a DHPS gene was used which was desensitised to lysine by at least one directed amino acid residue modification, preferably at the site of a in a wild-type enzyme conserved asparagine residue, and re-inserted into the cell of origin. In a preferred embodiment of the invention the replacement is located at or about the putative lysine binding site of DHPS, said site

in *Escherichia coli* located at around position 75-85, preferably at the site of a in a wild-type enzyme conserved asparagine residue.

The mutated gene is preferably put back in its original environment to which its expression, activity and interaction with other components is maximally adapted. Changing the endogenous enzyme by changing at least a single amino acid residue in the enzyme in its original host, such as a yeast, fungus, a plant or a bacterium, will also be far more acceptable to public and consumers than replacing the gene by a heterologous one.

In particular, the invention provides mutation of plant DHPS genes; genes encoding DHPS from various plant species have already been cloned, such as from *Nicotiana sylvestris* (Ghislain, M. et al. (1995) Plant J.8: 733-743), soybean (Silk, G.W. (1994) Plant Mol Biol 26: 989-993), wheat (Kaneko, T. et al. (1990) J. of Biol. Chem. 265: 17451-17455) and maize (Frisch, D.A. et al. (1991) Mol. Gen. Genet. 288: 287-293). Plant DHPS mutants with reduced sensitivity to lysine have been sought in order to use the mutated plant genes for increase of free lysine in plants. Direct selection of tobacco (*Nicotiana sylvestris*) UV-irradiated tissue cultures for selection to the lysine analog S-2-aminoethyl-L-cysteine (AEC) resulted in a dominant mutation that reduced lysine inhibition of DHPS and increased the concentration of free lysine in leaves and seed (Negrutiu, I. et al. (1984) Theor. Appl. Genet. 68: 11-20). However, no other plants with altered DHPS inhibition have been obtained by AEC selection. The tobacco mutated DHPS gene showed to contain a dinucleotide mutation located in a 10-amino acid-long region that presumably identifies the lysine-binding site of DHPS enzyme (Ghislain, M. et al. (1995) Plant J. 8: 733-743). The mutation resulted in the change of a conserved asparagine amino acid residue into a isoleucine residue. The change to obtain a di-nucleotide mutation needed for the conversion of asparagine into isoleucine by UV irradiation is extremely low, which could explain the fact that no other AEC resistant plants could be rescued.

Two other groups tried to mutate the conserved region involved in lysine binding of the plant DHPS enzymes in different ways. Shaver et al. ((1996) Proc. Natl. Acad. Sci. 93: 1962-1966) expressed the maize DHPS cDNA in an *E.coli dapA*$^-$ auxotroph, treated the cells with the mutagens ethylmethanesulfonate (EMS) and selected the cells on the lysine analogue AEC. They found several single nucleotide changes

resulting in amino acid replacements causing nearly complete lack of lysine inhibition. However, no mutation was observed at the conserved asparagine residue which is changed in the potato DHPS enzyme as provided herein. Introduction of gene constructs containing these mutated maize DHPS genes into maize cell cultures resulted into an increase of four times the lysine content in transformed cells compared to wild type cells in the case of mutant DHPS166av (Bittel, D.C. et al. (1996) Theor. Appl. Genet. 92: 70-77). The use of this mutated maize DHPS gene is also mentioned in U.S. Patent Application No. 5545545.

The group of Silk and Matthews ((1997) Plant Mol Biol. 33: 931-933) used the information of the above mentioned mutations in tobacco and maize to change conserved amino acids in the soybean DHPS enzyme. The cloned soybean DHPS cDNA was site-directed mutated by PCR. One mutant contained a single amino acid substitution at codon 104 (conserved asparagine was changed into isoleucine at the same location in the tobacco DHPS mutant), in another mutant a single amino acid substitution was performed at position 112 (conserved alanine was changed into valine at the same location in the maize DHPS mutant). A third mutant contained both mutations. All mutants show the same lysine insensitivity when expressed in *E.coli dapA*⁻ auxotroph cells. There are at present no data on expression of these mutant DHPS gene constructs in plant cells.

When the amino acid sequences of several bacterial and plant DHPS enzymes are compared, a number of conserved amino acids become clear (Shaver et al. ((1996) Proc. Natl. Acad. Sci. 93: 1962-1966). The invariant lysine residue at position 161 in E.coli is required for binding pyruvate. Furthermore, there are three amino acid residues very conserved in the 10 amino acid long stretch of the DHPS enzyme which is probably or putatively involved in the binding of lysine. The site comprises the conserved glycine at position 78 (position in E. coli), a conserved asparagine at position 80, and a conserved threonine at position 82. An amino acid residue which according to the invention is preferably replaced by cysteine is the conserved asparagine at position 80 (position 134 in potato DHPS and position 158 in corn). This same conserved amino acid residue was mutated in a tobacco DHPS mutant, however it was changed into an isoleucine in stead of a cysteine. The mutation of asparagine, or of an amino acid close to that asparagine, in cysteine is apparently

central to the increased lysine production of the transgenic organism, surprisingly, however, until now in none of cloned wild type and mutant bacterial, fungal or plant DHPS genes a cysteine residue is found at or around the (in wild type enzymes) conserved asparagine position.

The invention also provides a method for selecting a transformed host cell comprising a recombinant nucleic acid, or functional fragment thereof, encoding an enzyme dihydrodipicolinate synthase (DHPS), or functional fragment thereof, comprising culturing host cells in the presence of S-(2-aminoethyl)-L-cysteine and selecting a desired host cell for relative feedback insensitivity to S-(2-aminoethyl)-L-cysteine. Due to the combination of a randomised site-directed mutation method and selection for feedback insensitivity the invention provides an extremely insensitive DHPS mutant which, when for example expressed in potato plants, resulted in an accumulation of the free lysine content of more than 30% of the total free amino acid level in tubers. Increase of the lysine levels in potato is far more difficult than in, for instance tobacco, as can be seen by the experiments from Perl et al ((1993) Plant Mol Biol 19: 815-823) where expression of the bacterial DHPS (DapA) gene in potato resulted in far less lysine accumulation in potato than compared to expression of this gene in tobacco.

The invention also provides recombinant or isolated nucleic acid, or functional fragment thereof, encoding potato DHPS, or functional fragment thereof, preferably wherein said nucleic acid is at least 60%, preferably at least 75%, most preferably at least 90% homologous to a nucleic acid as shown in figure 2A. We mutagenised by PCR very specifically the conserved aspargine residue at position 134 in said potato DHPS enzyme into all other possible amino acid residues. The pool of mutant DHPS clones obtained in this way, containing all possible amino acid residues at position 134 was subsequently expressed in *E.coli dapA*$^-$ auxotroph cells and selected for lysine insensitivity with the lysine analogue AEC. The conserved amino acid residue that had to be mutated was selected by us, but the change to another amino acid residue was driven by the selection in *E.coli dapA*$^-$ cells.

The invention also provides a promotor or functional fragment thereof to drive expression of a DHPS gene as provided by the invention. Suitable promotors, such as plant-specific promotors are known in the art. In particular, the invention provides a

nucleic acid according to the invention further comprising a tissue-specific promotor or functional fragment thereof. A preferred promoter to drive expression of the DHPS enzyme according to the invention is the tuber-specific granule bound starch synthase (GBSS) promoter (Visser, R.G., Plant. Mol. Biol. 17:691-699, 1991). Other tuber-specific promoters can be used to drive DHPS expression like class I patatin promoter from *Solanum tuberosum* (Mignery, C.A. et al. (1988) Gene 62: 27-44; Wenzler, H.C. et al. (1989) Plant Mol.Biol. 12: 41-50), AGPase promoter (Muller-Rober, B., et al., Plant Cell 6:601-612, 1994), proteinase inhibitor II promoter from potato (Keil, M. et al. (1989) EMBO J. 8: 1323-1330), or the cathepsin D inhibitor promoter from potato (Herbers, K. et al. (1994) Plant Mol. Biol. 26: 73-83). Also other tissue-specific promoters can be used like the fruit-specific promoter from tomato polygalacturonidase gene (Grierson, D. et al. (1986) Nucl.Acids Res. 14: 8595-8603), or the seed-specific phaseoline promoter from bean (Sengupta-Gopalan, C. (1985) Proc.Natl.Acad.Sci.USA 82: 3320-3324). Other promoters that can be used are inducible promoters, like the light inducible promoter derived from the pea rbcS gene (Coruzzi G. et al. (1984) EMBO J. 3: 1671-1679) and the actine promoter from rice (McElroy, D. et al. (1990) The Plant Cell 2: 163-171).

The promoter is in general to be found in the 5' region of each of the gene. Since the organelle in which lysine biosynthesis takes place in higher plants is the plastid, the gene construct comprises also a DNA sequence coding for a transit peptide which is involved in the translocation of the protein from the cytosol into the plastids (Van den Broeck, G. et al. (1985) Nature 313: 358-363; Schreier, P.H. et al. (1985) EMBO J. 4: 25-32). The chloroplast targeting signal can either naturally be present in a DHPS gene originating from a plant or has to be added when the DHPS gene originates from bacterial, fungal or algal organisms. This DNA sequence encoding a chloroplast transit peptide fused to a DNA sequence coding for DHPS, will on expression produce a fused DHPS/transit peptide chimeric protein in the cytoplasm of the transformed plant cell, which will be transported to the plastids, where increased production of lysine is thereby obtained.

In a particular embodiment of the invention, the 3' end of the DNA sequence coding for the transit peptide is fused to the DNA sequence encoding DHPS, which is fused to a transcription termination DNA signal. This termination signal comprises a

3' transcription termination and a mRNA polyadenylation signal. Termination signals present at the 3' flanking region of any cloned gene can be used, e.g. from the pea rbcS gene, the bean phaseoline gene, or the nopaline synthase gene derived from the Ti plasmid of *Agrobacterium tumefaciens*. A preferred terminator sequence originates from the 3' flanking region of the octopine synthase gene from the Ti plasmid of *Agrobacterium tumefaciens* (Figure 3; Greve, H.D. et al. (1983) J.Mol.Appl.Genet. 1: 499-511).

The invention also provides a vector comprising a nucleic acid according to the invention. For example, an expression vector can be of the class of high copy number pUC or pBR322 derived plasmids, which can be used for direct transformation methods, like electroporation, particle bombardment or poly-ethylene-glycol mediated transformation procedures. Alternatively, expression constructs are included on a Ti plasmid derived, so-called binary vector, like pBINPLUS (Van Engelen, F.A. et al. (1995) Transgenic Research 4: 288-290). The Ti plasmids can be propagated in *Agrobacterium tumefaciens*, from which the inserted DNA fragments between the left and right border are transferred to the plant cell.

The expression vector in which the gene construct as provided by the invention, such as a chimeric gene construct, is cloned, is introduced into host cells such as fungal, bacterial, algal or plant cells. The introduction is realised using any kind of transformation protocol capable of transferring DNA; for plants several protocols have been developed for to either monocotyledonous or dicotyledonous plant cells. For example: transformation of plant cells by direct DNA transfer via electroporation (Dekeyser, R.A. et al. (1990) The Plant Cell 2: 591-602), via PEG precipitation (Hayashimoto, A. et al. (1990) Plant Physiol. 93: 857-863) or via particle bombardment (Gordon-Kann, W.J. et al. (1990) The Plant Cell 2: 603-618), and DNA transfer to plant host cells via infection with Agrobacterium. A preferred method is via infection of plant cells with *Agrobacterium tumefaciens* (Horsch, R.B. et al. (1985) Science 227: 1229-1231; Visser, R.G.F. (1991) Plant Tissue Culture Manual B5 (ed. by K. Lindsey): 1-9, Kluwer Acad. Publishers, The Netherlands). The methodology used here for in the detailed description for potato or grass is also provided to improve many other crop plants such as for example transgenic sugar beet, carrot, cassave, canola, alfalfa, legumes, and gramineae species like rice, maize, wheat, sorghum,

barley and grasses like *Lolium perenne*, in a preferred embodiment of the invention, said plant comprises a tuber, such as a potato, from which lysine rich plant material can be obtained by tissue specific expression as provided herein. Other specific tissues wherein the DHPS-gene preferentially can be expressed comprise roots and seeds.

Transformed plants are for example selected by resistance to kanamycin or other antibiotics like hygromycin, or herbicides like bialaphos or phosphinotricin.

Alternatively, desired transgenic plants or transgenic host cells (from which plants can be obtained) with a high lysine content are selected by cultivation in the presence of a lysine analogue, such as S-(2-aminoethyl)-L-cysteine (AEC), without selection on resistance to antibiotics or herbicides, as described above where E. coli host cells were selected. In a preferred example, the tissue-specific promoter that is used to drive DHPS expression is activated during the AEC selection. For example, the tuber-specific granule bound starch synthase (GBSS) promoter is induced by high levels of sucrose [Visser, R.G.F. et al. (1991) Plant Mol. Biol. 17:691-699), as is the AGPase promoter (Muller-Rober, B. et al. (1990) Mol. Gen. Genet. 224: 136-146) and the class I patatine promoter (Wenzler, H.C. et al. (1989) Plant Mol. Biol. 12: 41-50). Other tissue-specific promoters like the seed-specific phaseoline promoter from bean and the zein promoter from maize are induced during AEC selection by addition of ABA (Muller, M. et al. (1997) Plant J. 12 (2): 281; Bustos, M.M. et al. (1998) Plant Mol. Biol. 37(20): 265). No marker genes except for the DHPS gene thus have to be introduced.

The invention furthermore provides a method to obtain plant material with relative high lysine content comprising harvesting at least a part of a plant according to the invention. Harvesting crop plants and obtain specific plant material, such as seeds, leaves, stems, roots, tubers or fruits, is a skill known in the art, allowing to obtain plant material from a plant according to the invention. The invention thus provides a method to increase lysine content of food or feed with relative low lysine content comprising adding plant material according to the invention to said food or feed. Lysine rich food or feed is also provided, and last but not least, it is in itself possible to use the lysine-enriched plant material according to the invention, such as

lysine enriched potatoes, grasses, seeds or fruits as food or feed. The invention is further explained in the detailed description without limiting the invention.

Detailed description.

Example 1. Cloning of the potato DHPS cDNA

DNA isolation, subcloning, restriction analysis and DNA sequence analysis is performed using standard methods (Sambrook, J. et al. (1989) Molecular Cloning. A laboratory manual, Cold Spring Harbor Laboratory Press; Ausubel, F.M. et al. (1994) Current protocols in molecular biology, John Wiley & Sons).

For the isolation of the *Solanum tuberosum* cv Kardal gene encoding dihydrodipicolinate synthase (DHPS) a cDNA library of mRNA prepared from young tubers was constructed in the vector λTriplEx (Clotech). A specific 600 basepairs DHPS DNA fragment for screening the cDNA library via heterologous hybridisation was cloned by the reverse transcriptase polymerase chain reaction (RT-PCR) on total RNA isolated from *Arabidopsis thaliana* and subsequently ligated into vector pMOSBlue (Amersham), resulting in pAAP15. To this purpose synthetic oligonucleotides based on the sequence of the *Arabidopsis thaliana* DHPS gene were used (Vauterin & Jacobs, 1994).

A single plaque from the cDNA hybridising to pAAP15 was selected and the 1190 basepairs DNA insert completely sequenced (Figure 2A). It was concluded to encode a dihydridipicolinate synthase. The DNA fragment encompassing the complete coding sequence was cloned in the cloning vector pBluescript SK(+) (Stratagene) via a PCR-based strategy to enable the expression in *Escherichia coli* of a β-galactosidase-DHPS fusion protein under the control of a *lac* promoter. The resulting plasmid was designated pAAP57. This gene construct was able to complement *E. coli* AT997 (Yeh et al., 1988), a DHPS deficient strain, allowing it to grow on minimal medium in the absence of DL-α,ε-diaminopimelic acid.

Example 2. Mutagenesis to create a lysine insensitive DHPS gene

In order to create a feedback insensitive DHPS, nucleotides in pAAP57 corresponding to the evolutionary conserved amino acid residue 134 (asparagine) were changed at random via a PCR-based approach (QuickChange site directed mutagenesis, Stratagen), resulting in a population of plasmids encoding DHPS enzymes with different amino acids residues at position 134. Following transformation of *E. coli* AT997 with this plasmid population, selection for feedback insensitivity was done in the presence of 1 mM of the lysine analogue S-(2-aminoethyl)-L-cysteine (AEC). Several AEC resistant colonies were picked and the DHPS coding region of their plasmid directing AEC resistance sequenced (Figure 2B). The change of AAC into either TGT or TGC resulted in the corresponding change of the asparagine residue at DHPS position 134 into a cysteine residue. The mutant DHPS encoding DNA fragment (designated DHPS-134nc1) was used for the expression in potato plants.

Example 3. Chimeric gene construct with the mutant potato DHPS gene

The chimeric gene containing the mutant DHPS gene was constructed by subcloning DHPS cDNA from the pTriplex vector (pAAP42) first in pCR-Script SK(+) (pAAP55) and from this vector as a XbaI–Eco RI fragment in the pBluescript SK vector digested with XbaI–EcoR (pAAP57). With this clone the mutagenesis was performed, resulting in clone pAAP57-134nc1. At the 5'end the mutated DHPS cDNA was fused to a HindIII–SalI fragment of the 800 bp long GBSS promoter fragment (Visser et al. ibid). Downstream of the mutant DHPS sequence the termination signal of the nopaline synthase gene from *Agrobacterium tumefaciens* was inserted (Greve, H.D. et al. (1983) J.Mol.Appl.Genet. 1: 499-511) as an SstI–EcoRI fragment. The complete chimeric gene was subcloned into the HindII–EcoRI sites of pBINPLUS (Van Engelen, F.A. et al. (1995) Transgenic Research 4: 288-290) (pAAP105, Figure 3).

Example 4. Introduction of the chimeric gene into potato

4.1 Transformation of potato plants

The binary vector pAAP105 was used for freeze-thaw transformation of *Agrobacterium tumefaciens* strain AGLO (Höfgen, R. and Willmitzer, L. (1988) Nucl.Acids Res. 16: 9877). Transformed AGLO was subsequently used for inoculation of potato (*Solanum tuberosum*, variety Kardal) stem explants as described by Visser (Visser, R.G.F. (1991) Plant Tissue Culture Manual B5 (ed. by K. Lindsey): 1-9, Kluwer Acad. Publishers, The Netherlands). After shoot and root regeneration on kanamycin-containing media plants were put in soil and transferred to the greenhouse. Plants regenerated (on kanamycin-free media) from stem explants treated with the Agrobacterium strain AGLO lacking a binary vector served as a control.

4.2 Selection of transformed plant cells on AEC

In stead of selection of transformed plant cells on kanamycine, as described by Visser (Visser, R.G.F.(1991) in : K. Lindsey (ed) Plant Tissue Culture Manual B5: 1-9, Kluwer Acad Publishers) mentioned above (4.1), the transformed plant cells containing a mutant DHPS gene can also be selected on the lysine analogue AEC. After inoculation of for example potato stem explants with the transformed (AGI 0) strain, explants are in general first cultured on medium without AEC. After a short period, for example 12-20, such as 16, days they are transferred to medium with 0.1 mM AEC and a sufficient concentration (e.g. 5%) sucrose. When the first primordia are visible the explants are transferred to 0.025 mM AEC and sucrose. Root induction is performed in the absence of AEC/sucrose-selection.

4.3 In vitro tuber formation

In order to induce in vitro tuberisation, nodal cuttings (about 4-5 cm long) of transformed potato plantlets are placed vertically in solid Murashige and Skoog

medium (Murashige, T. and Skoog, F. (1962) Physiol.Plant. 15: 473-497) supplemented with 10% (w/v) sucrose, 5 TM BAP. The cultures are maintained at 19°C in the dark. After 14 days microtubers of 4 mm in diameter are harvested and analysed for free lysine and threonine content, and for AK and DHPS activity. The protein content and the protein composition are analysed as described below.

4.4. Selection of transformed shoots with high DHPS* expression on aminoethylcysteine (AEC)

In stead of selection on kanamycin after transformation with the pAAP105 construct by Agrobacterium inoculation (see example 4.1), the transformed shoots can be selected by rooting on a medium containing a lysine analogue. The mutant DHPS gene can be used as a selection marker because plant cells with a high content of lysine can grow on toxic lysine analogues, such as aminoethylcysteine (AEC). Due to the feedback-insensitivity and resulting high endogenous lysine levels, the toxic analogue is diluted out and cannot bind to the lysine binding site in the mutant enzyme.

After transformation of potato stem explants (see 4.1) with the pAAP105 containing Agrobacterium strain, the explants were put on shoot regeneration medium without kanamycin. When shoots appeared they were either put directly on rooting medium containing 0.06 mM AEC with 50 g/l sucrose (for induction of the GBSS promoter), or were rooted first on rooting medium without AEC and afterwards were rooted again on medium containing 0.06 mM AEC and 50 g/l sucrose. Shoots that were able to root on this concentration AEC were cut again for rooting on medium containing 0.1 mM AEC and 50 g/l sucrose. The criteria for rooting resistance on 0.06 and 0.1 mM AEC are the following:

- roots should appear at the cutting edge of the shoot
- cutting edge should not be yellow-brownish and rotten
- root tips should be light coloured

The transformation frequency leading to transgenic plants with eventually high lysine levels in the tubers is about 0.5%.

Example 5. DHPS modification of perennial ryegrass (*Lolium perenne* L.)

Plant materials

Embryogenic suspension cultures from different *Lolium perenne* cultivars, a.o. the cultivars Moronda and Aurora, are initiated directly from mature, seed-derived embryos or from embryogenic callus cultures obtained from immature inflorescence segments, essentially as described by Creemers-Molenaar et al., *Plant Science* 63:167-176 (1989). For the direct approach, seeds are sterilized in 10% hypochlorite, rinsed and soaked for two days in sterile tap water and sterilized for a second time. After rinsing thoroughly, mature embryos are dissected from 40 seeds, chopped and transferred to 5 ml of MS10 medium (i.e. Murashige and Skoog basal salts and vitamins supplemented with 10 mg/l 2,4-D and 3% sucrose at pH 5.8) in a 60 ml plastic specimen container (Thovadex). This in several replicates. Embryogenic callus is induced on immature inflorescence segments of greenhouse grown plants after sterilization with 5% hypochlorite and rinsing with sterile water. The basal parts of the inflorescences are cut into 2 segments of 1-2 mm long and placed on MSt5 medium (i.e. MS basal salts and vitamins supplemented with 0.4 mg/l (=extra) thiamin-HCl, 5 mg/l 2,4-D and 3% sucrose) solidified with 0.8% Daichin agar. Culture is in the dark at 25 °C. After 4-8 weeks compact, embryogenic callus is excised from the explants of 2-5 plants (i.e. genotypes). The calli are mixed, chopped with a scalpel and transferred in 0.1 g FW aliquots to 5 ml of MS10 in specimen containers. From this point on, the cultures of both origins are treated identically. The cultures are incubated on a rotary shaker (140 revs./min.) in continuous indirect light (200-400 lux) at 25 °C.

After 10 days the medium is replaced with MS5 (= as MS10 but with 5 mg/l 2,4-D). Once a week 2 ml of fresh medium is added to the cultures until a final volume of 15 ml is reached. Subsequently, the cultures are transferred to new 190 ml transparent, polystyrene containers (Greiner) and 5 ml fresh medium is added. For further experimentation well-proliferating, finely-dispersed cultures are selected and maintained by weekly subculturing 2.5 g FW material in 20 ml fresh MS5. Incubation is in the dark under continuous shaking at 120 revs./min. at 25 °C.

The regeneration potential of the cultures is determined by placing 0.5-1.0 g FW material on solid MS0 (0 mg/l 2,4-D; 0.8% agar). After culturing in the dark at 25 °C for the first 2 weeks the calli are placed in dimmed light (500-1000 lux) for 16 hours/day for the next 2 weeks. Finally after transfer to fresh MS0, they are placed in 4000 lux, 16 hours/day and the number of calli producing shoots is scored after 4 weeks.

Transformation

Three days after subculture, 0.25 g FW callus material consisting of cells in log-phase, is evenly dispersed onto the surface of a 42 mm diameter Whatman filter disc (Schleicher & Schuell #604). Subsequently, the filters are moistened by the addition of 0.5 ml fresh culture medium and they are placed onto culture medium solidified with 0.2% gelrite and left overnight at 25 °C in the dark. The next day the filters carrying the perennial ryegrass suspension material are used for biolistic gene transfer using the PDS1000-He particle gun (BioRad). 0.375 mg Gold particles with an average diameter of 1 µm were coated with 0.625 µg DNA of plasmid pAAP205, which is a derivative of the plasmid pAAP105 mentioned in Example 3 lacking T-DNA borders and with the *npt*II gene replaced by the *hpt* gene for selection of transgenic perennial ryegrass cells. For the coating, 50 µl (= 3 mg) of washed particles are suspended thoroughly by vortexing. Subsequently, 5 µl plasmid DNA (concentration 1 µg/µl) and 50 µl 2.5M $CaCl_2$ are added and vortexed for 10 seconds. Then, 20 µl 0.1M free-base spermidine is added and mixed by vortexing for 2 seconds. The mixture is centrifuged for 5 seconds and the supernatant is removed, after which 250 µl ethanol 96% is added followed by vortexing for 1 minute. After washing with ethanol once, the particles now coated with DNA are resuspended in 60 µl ethanol 96% and kept on ice until use. Bombardment can be performed at pressures ranging from 1100 psi to 2200 psi, but in this example particularly a pressure of 1800 psi is used while the dish containing the filters is placed at a distance of 9 cm. After biolistics the filters are incubated on the culture medium in the dark at 25 °C for 24 hours before they are transferred to selection medium. For selection, the filters are first placed on culture medium MSt5 solidified with 0.2% gelrite containing 80 mg/l hygromycin; after 1 week the filters are transferred to selection medium containing 150 mg/l hygromycin. So far, this is essentially as described earlier (Van der Maas et

al., *Plant Mol. Biol.* 24:401-405 [1994]). Actively growing calli are individually transferred to fresh selection medium (150 Hyg.) after 4 weeks. Following this second round of selection surviving calli are placed on regeneration medium supplemented with 50 mg/l hygromycin. Transgenic perennial ryegrass plants are obtained and collected after 8 weeks. They are maintained in tubes containing half strength MS0 (Creemers-Molenaar et al., *Plant Science* 57: 165-172 [1988]) and subsequently characterized molecularly by PCR and Southern hybridization analysis and biochemically by amino acid, enzyme activity and protein analysis as described in Example 6 - 9 to confirm presence and expression of the newly introduced gene constructs.

Example 6. Analysis of free amino acid content in transgenic plants

Tissue (0.5-1.0 gram) was homogenised with mortar and pestle in 2 ml 50 mM Pi-buffer (pH 7.0) containing 1 mM dithiothreitol. Nor-leucine is added as an internal standard. Free amino acids were partly purified by extraction with 5 ml of a water:chloroform:methanol mixture (3:5:12). Water phase was collected and the remaining re-extracted twice. After concentration by lyophilisation to 3 ml, a 20 µl sample was analysed by HPLC using a cation-exchange column with post-column ninhydrine derivatisation of the amino acids detected at 570 and 440 nm (BIOCHROM 20, Amersham Pharmacia biotech). Figure 4 shows the lysine levels as the percentage of the total amino acids in tubers in 27 untransformed potato plants and in 30 potato plants containing the mutated DHPS gene construct pAAP105. The lysine levels increase from 2-2.5% in the control wild type tubers to a maximum percentage of 30 in the transformed tubers, resulting in lysine being a 'bulk' amino acid in stead of a 'low level' essential amino acid .

Example 7. Analysis of DHPS enzyme activity in transgenic plants

Microtubers or mature tubers were homogenised with mortar and pestle in an equal volume of cold 100mM Tris-HCl pH 7.5 containing 2 mM EDTA, 1.4% sodium ascorbate, 1mM phenylmethylsulphonilfluoride and 0.5 Tg/ml leupeptin. Following 5 min. centrifugation (16,000 g at 4^{0}C) the supernatant is collected. DHPS activity was measured using the O-aminobenzaldehyde (O-ABA) method of Yugari and Gilvarg (Yugari, Y. and Gilvarg, C. (1965) J.Biol.Chem. 240: 4710-4716). As expected, AEC sensitivity of transformed was reduced, when comparing the wild type and mutant DHPS activity in respectively control (untransformed) and transformed potato tubers, the latter showed DHPS acitivity even under increased levels of AEC.

Example 8. DNA analysis of transgenic plants

Genomic DNA was isolated from potato tubers of plants grown in the green house. Lyopholised tissue was ground in liquid nitrogen and added to 15 ml extraction buffer (100mM Tris.HCl, pH 8, 500mM NaCl, 50mM EDTA and 10mM ß-mercaptoethanol) and 2 ml 10% SDS. After incubation at 65 °C for 20 min, 5 ml of 5M KAc were added and incubated on ice for 15 min. After centrifugation, filtration of the supernatant, and precipitation with 15 ml iso-propanol, the pellet was resuspended in 400 µl TE and treated with 1 µg RNAse. The DNA was subsequently purified with CTAB (N-Cetyl-NNN tri-methylammonium bromide) extraction by adding 400 µl of CTAB buffer (200mM Tris-HCl pH 7.5, 50mM EDTA, 2mM NaCl and 2% CTAB) and incubating at 65 0C for 15 min. After extraction with 800 µl chloroform/isoamyl alcohol 24:1, the DNA was precipitated with 800 µl iso-propanol. The pellet was washed with 70% ethanol and resuspended in TE. DNA. After digestion with restriction enzymes, the DNA was size-fractionated on an agarose gel and blotted onto Hybond-N$^+$ (Amersham). Random-primer-labeled probe was derived from the DHPS cDNA (from position 337 to 811, Figure 2). Filters were hybridised at 65 ^{0}C in 10 % dextran sulphate, 1% SDS and 1M NaCL for 16 to 20 hr. and washed subsequently at 55 ^{0}C and 60 ^{0}C with 2x SSC, 0.1 % SDS, and at 65 ^{0}C with 0.1x SSC, 0.1% SDS.

Example 9. Expression analysis of transgenic plants on RNA level

Total RNA was isolated from potato tubers grown in the greenhouse according to the protocol described by Ausubel et al. (1994). RNA was size-fractionated on formaldehyde agarose gels and blotted onto Hybond-N^+ (Amersham). Blots were briefly stained to ensure that equal amounts of RNA were present. Random-primer-labeled probe was derived from the DHPS cDNA (from position 337 to 811, Figure 2). Filters were hybridised at 65 ^{0}C in 10 % dextran sulphate, 1% SDS and 1M NaCL for 16 to 20 hr. and washed subsequently at 55 ^{0}C and 60 ^{0}C with 2x SSC, 0.1 % SDS, and at 65 ^{0}C with 0.1x SSC, 0.1% SDS.

Figure legends

fig.1:

A diagram of the aspartate family biosynthetic pathway. Only the major key enzymes are indicated. Curved arrows represent feedback inhibition by the end product amino acids DHPS, dihydrodipicolinate synthase; HSD, homoserine dehydrogenasse; TDH, theonine dehydratase.

fig.2:

(A) Nucleic acid fragment encoding a DHPS isolated from potato. The derived amino acid sequence is presented using the 1-letter code.

(B) Nucleic acid sequence and derived amino acid sequence of a lysine binding domain of the mutated DHPS (DHPS-134nc.)

fig.3:

Schematic representation of the T-DNA region of plasmid pAAP105, encompassing a mutated dihydrodipicolinate coding region (DHPS) under control of the granule bound starch synthase promoter (P-GBSS) and as selection maker the neomycinephosphotransferase 11 coding region (NPT11) under control of the nopaline synthase promoter(P-NOS);I-NOS nopaline synthase transcription terminator.

fig.4:

Free lysine concentration(micromol/g fresh weight) of tubers from tubers derived from different, independent transformant or control plants. Plants were grown in pots in the green house until maturity. After harvesting tubers were dried for one week before analysis. About 10 gram of tuber material was ground in 50 ml of a 1 mM dithiothreitol (DTT) solution. Two ml of the resulting suspension was extracted with a menthanol: water: chloroform mixture(12:3:5). After concentration to 0.5 ml, 25µl is used for analysis on a Biochrom 20(Amersham-Phamacia) lithium-based ionexchange amino acid analysis system. Samples were spiked with L-Norleucine before grinding, to allow corrections.

fig. 5.

Southern blot DNA analysis of several transformed potato plants.

Genomic DNA was isolated from several independent transformed potato plants and untransformed plants, and digested with either HincII or with a combination of HincII and BamHI. After separation and blotting, the DNA fragments were hybridised with a radio-labelled DHPS probe. The untransformed control plants (C) show only hybridisation of the endogenous DHPS fragments. On top of those bands the transformed plants show one (lane 7) or more extra hybridising bands (e.g. lane 5) revealing the insertion of one or more copies of the GBSS-DHPS* construct. Abbreviations: C, control plants; M, molecular size marker.

Fig. 6.

Northern blot RNA analysis of the tubers of several transformed potato plants.

Total RNA was isolated from tubers of several transformed plants (lanes 1-15) and from control untransformed plant (lane C). RNA was separated by gel-electrophoresis and blotted on nylon. The blot was hybridised with a radio-labelled DHPS DNA probe. Control RNA from an untransformed plant shows hybridisation of the endogenous DHPS RNA (lane C). RNA from several transformed plants show hybridisation which is equal to the background DHPS hybridisation (e.g. lanes 4 and 9). Most of the transformed plants show a strong hybridisation to the expressed exogenous DHPS* RNA (e.g. lanes 10-12).

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Claims

1. A recombinant or isolated nucleic acid, or functional fragment thereof, encoding an enzyme dihydrodipicolinate synthase (DHPS), or functional fragment thereof, having been provided with a mutation leading to the replacement of at least one single amino acid residue with a cysteine residue.

2. A nucleic acid according to claim 1 wherein said amino acid replacement is located at or about the putative lysine binding site of DHPS, said site in *Escherichia coli* located at around position 75-85.

3. A nucleic acid according to claim 1 or 2 wherein said single amino acid residue comprises an asparagine residue.

4. A nucleic acid according to anyone of claims 1 to 3 wherein in potato DHPS said asparagine residue is located at position 134.

5. A nucleic acid according to anyone of claims 1 to 4 wherein said DHPS is of bacterial, fungal, algal or plant origin.

6. A nucleic acid according to claim 5 wherein said plant is a potato.

7. A recombinant or isolated nucleic acid, or functional fragment thereof, encoding potato DHPS, or functional fragment thereof.

8. A nucleic acid according to claim 7 wherein said nucleic acid is at least 90% homologous to a nucleic acid as shown in figure 2A.

9. A nucleic acid according to anyone of claims 1 to 8 further comprising a tissue-specific promotor or functional fragment thereof.

10. A nucleic acid according to claim 9 wherein said promotor is derived from the tuber-specific granule bound starch synthase (GBSS) promotor.

11. A vector comprising a nucleic acid according to anyone of claims 1 to 10.

12. A host cell comprising a nucleic acid according to anyone of claims 1 to 10 or a vector according to claim 11.

13. A host cell according to claim 12 comprising a plant cell.

14. A host cell according to claim 13 wherein said plant cell is derived from a potato.

15. A method for selecting a transformed host cell comprising a recombinant nucleic acid, or functional fragment thereof, encoding an enzyme dihydrodipicolinate

synthase (DHPS), or functional fragment thereof, comprising culturing host cells in the presence of S-(2-aminoethyl)-L-cysteine and selecting a desired host cell for relative feedback insensitivity to S-(2-aminoethyl)-L-cysteine.

16. A method according to claim 15 wherein said nucleic acid comprises a nucleic acid according to anyone of claims 1 to 10.

17. A host cell obtainable with a method according to claim 15 or 16.

18. A plant comprising a host cell according to claim 14 or 17.

19. A plant according to claim 18 comprising a crop plant.

20. A plant according to claim 18 or 19 comprising a tuber.

21. A tuber derived from a plant according to claim 20.

22. A method to obtain plant material with relative high lysine content comprising harvesting at least a part of a plant according to claim 18.

23. Plant material obtainable by a method according to claim 22.

24. A method to increase lysine content of food or feed with relative low lysine content comprising adding plant material according to claim 23 to said food or feed.

25. Food or feed obtainable by a method according to claim 24.

26. Food or feed comprising plant material according to claim 24.

Figure 1

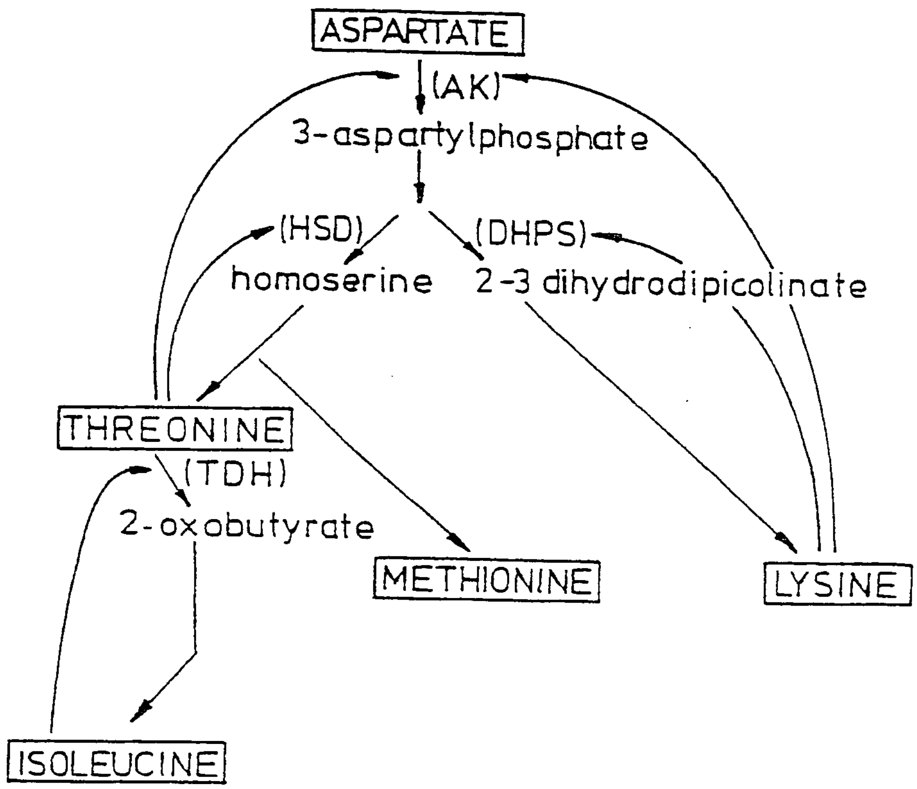
ASPARTATE
(AK)
3-aspartylphosphate
(HSD)
(DHPS)
homoserine
2-3 dihydrodipicolinate
THREONINE
(TDH)
2-oxobutyrate
METHIONINE
LYSINE
ISOLEUCINE

Figure 2A

```
CCGATAGGGA GAAATGTCAT CCTCAATTAC AAATTGCTGC CATTTCTATG    50
             M  S     S  S  I  T    N  C  C    H  F  Y
TTGAACGTGC CAGAACAAAC AGGAGAACTG CAACATGGAA GTCACCAAGA   100
V  E  R  A    R  T  N    R  R  T    A  T  W  K    S  P  R
GCAGCAGTAA TTCCATGTAT CCACCTCCCG ATGCGCAGTA ATGAAGTTAA   150
 A  A  V    I  P  C  I    H  L  P    M  R  S    N  E  V  K
AAATAGGACA TTTGCTGAGG ACATAAAAAA ACTTCGATTG ATTACTGCAA   200
 N  R  T    F  A  E    D  I  K  K    L  R  L    I  T  A
TTAAAACCCC ATATCTACCA GATGGAAGAT TCGATCTTGA GGCTTATGAT   250
I  K  T  P    Y  L  P    D  G  R    F  D  L  E    A  Y  D
GCCTTAGTAA ATTTGCAAAT TGAAAATGGT GTCGAGGGTG TGATTGTTGG   300
 A  L  V    N  L  Q  I    E  N  G    V  E  G    V  I  V  G
TGGCACTACA GGTGAAGGTC AATTGATGAG CTGGGATGAA CACATCATGC   350
 G  T  T    G  E  G    Q  L  M  S    W  D  E    H  I  M
TCATTGGTCA TACCATCAAT TGTTTTGGTG GATCAATTAA AGTCATTGGA   400
L  I  G  H    T  I  N    C  F  G    G  S  I  K    V  I  G
AACACAGGAA GCAACTCCAC GAGGGAAGCA ATCCATGCGA CAGAACAAGG   450
 N  T  G    S  N  S  T    R  E  A    I  H  A    T  E  Q  G
ATTTGCTGTA GGTATGCATG CAGCTCTTCA CATTAATCCT TACTATGGGA   500
  F  A  V    G  M  H    A  A  L  H    I  N  P    Y  Y  G
AGACCTCCAT AGAGGGTTTG ATTTCTCACT TTGACAGTGT GCTCCCTATG   550
K  T  S  I    E  G  L    I  S  H    F  D  S  V    L  P  M
GGCCCTACTA TCATTTACAA TGTGCCATCA CGAACTGGTC AAGACATTCC   600
 G  P  T    I  I  Y  N    V  P  S    R  T  G    Q  D  I  P
CCCTCATGTA ATCCAGACAT TAGCAAAGAG CCCTAACCTT GCTGGTGTCA   650
  P  H  V    I  Q  T    L  A  K  S    P  N  L    A  G  V
AAGAGTGTGT AGGAAATGAT AGAGTGGAGC AGTATACAAG TAATGGGTTG   700
K  E  C  V    G  N  D    R  V  E    Q  Y  T  S    N  G  L
GTTGTGTGGA GTGGGAATGA TGATGAATGC CATGACTCAA GGTGGCATCA   750
 V  V  W    S  G  N  D    D  E  C    H  D  S    R  W  H  H
TGGTGCTACG GGAGTTATTT CTGTCACCAG CAACTTGGTT CCTGGTCTGA   800
  G  A  T    G  V  I    S  V  T  S    N  L  V    P  G  L
TGCGTGAACT AATGTTTGGA GGAAATAACC CTGTTTTGAA CTCAAAGCTA   850
M  R  E  L    M  F  G    G  N  N    P  V  L  N    S  K  L
ATGCCCTTGG TTGAATGGTT GTTTCATGAG CCAAACCCCA TTGCTCTGAA   900
 M  P  L    V  E  W  L    F  H  E    P  N  P    I  A  L  N
TACAGCTTTA GCTCAACTTG GGGTTGTGAG GCCTGTTTTT CGCCTTCCCT   950
  T  A  L    A  Q  L    G  V  V  R    P  V  F    R  L  P
ATGTTCCACT TACAAAGGCA AAGAGAGAGG AGTTTGTGAA GATTGTTGAG  1000
Y  V  P  L    T  K  A    K  R  E    E  F  V  K    I  V  E
AAAATTGGAC AGGAAAACTT TATTGGGGAA AGAGATGTCC AAGTTTTGGA  1050
 K  I  G    Q  E  N  F    I  G  E    R  D  V    Q  V  L  D
TGATATTGAT TTTTTTTTTG GTTGGTAGGT ATTAGTTTTT CCCCAATTGG  1100
  D  I  D    F  F  F    G  W
TATTGAGAAG TAAGTTTTTG TTGTTAGCGG CTTAATTTGA AAAATTTCTT  1150
GACATGAAGA TATCTTCTTT ATGTTGTTTT TTAAAAAAAA             1190
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Figure 2B

```
AGTCATTGGA AACACAGGAA GCTGTTCCAC GAGGGAAGCA ATCCATGCGA   440
  V  I  G    N  T  G    S  C  S  T    R  E  A    I  H  A
```

Figure 3

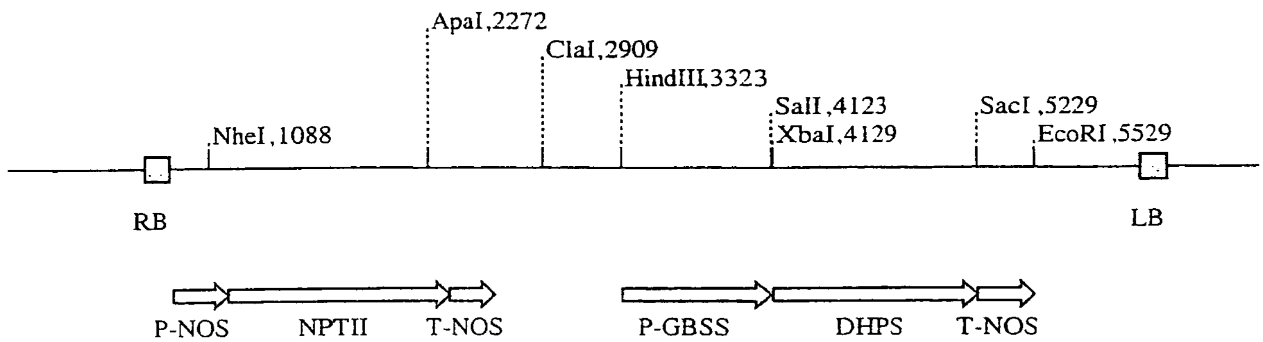
ApaI,2272
ClaI,2909
HindIII,3323
SalI,4123
XbaI,4129
SacI,5229
EcoRI,5529
NheI,1088
RB
LB
P-NOS
NPTII
T-NOS
P-GBSS
DHPS
T-NOS

Figure 4

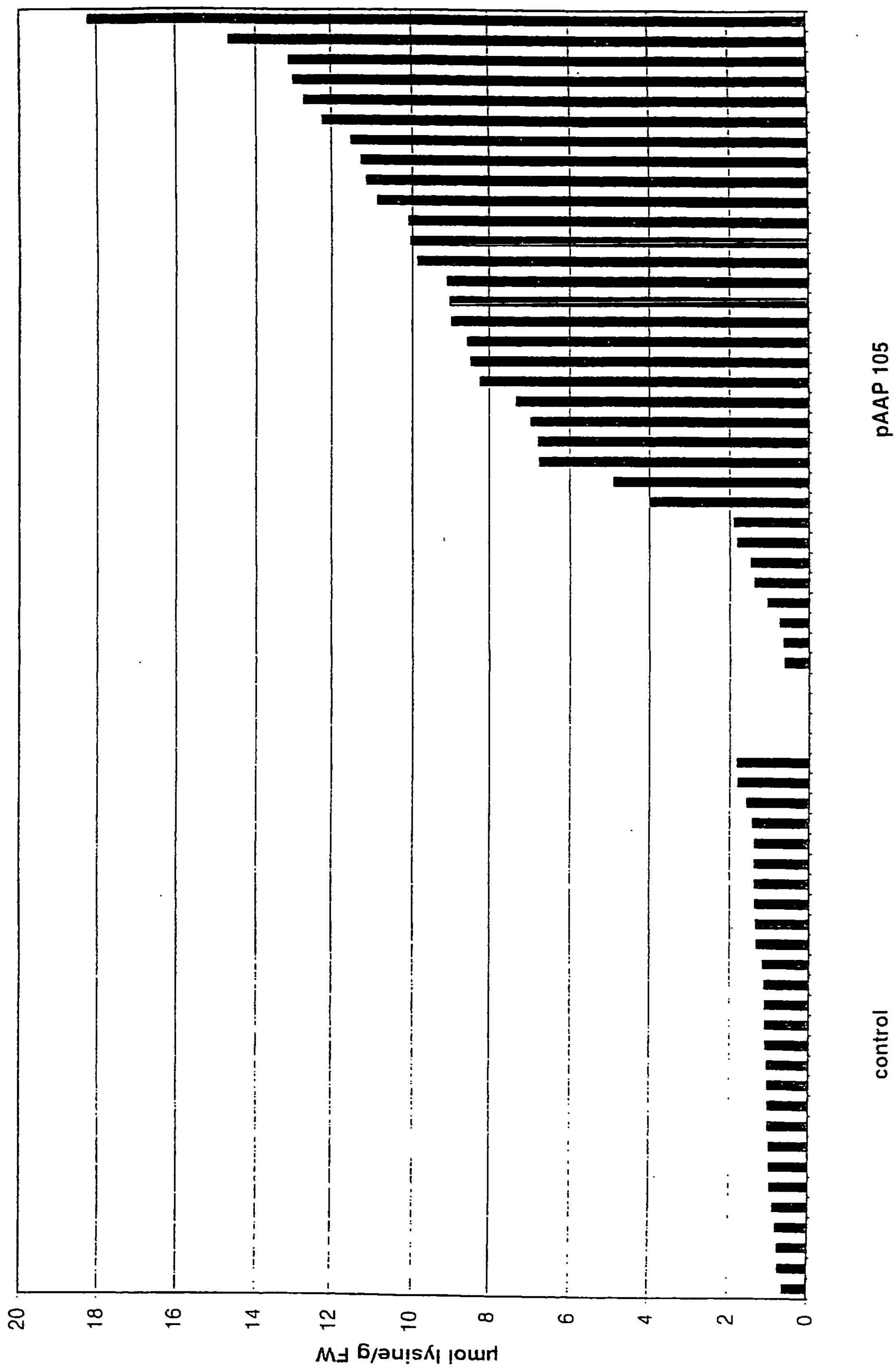

Figure 5

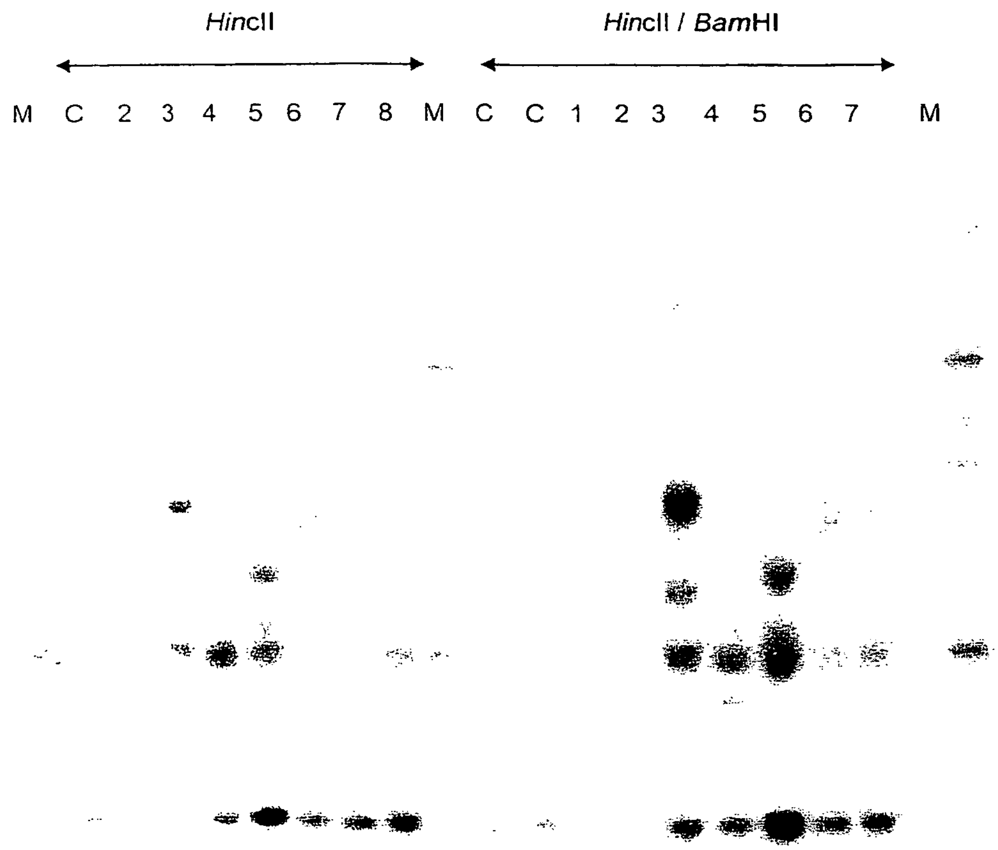

Figure 6

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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99204520.3 24 December 1999 (24.12.1999) EP

(71) Applicant *(for all designated States except US)*: **COÖPERATIEVE VERKOOP- EN PRODUCTIEVERENIGING VAN AARDAPPELMEEL EN DERIVATEN AVEBE B.A.** [NL/NL]; Beneden Oosterdiep 27, NL-9641 JA Veendam (NL).

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(74) Agent: **PRINS, A., W.**; Vereenigde, Nieuwe Parklaan 97, NL-2587 BN The Hague (NL).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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WO 01/48230 A3

(54) Title: MODIFIED DHPS GENES

(57) Abstract: The invention relates to modifications of biosynthesis key enzymes leading to essential amino acid accumulation in cells. A recombinant or isolated nucleic acid, or functional fragment thereof, encoding an enzyme dihydrodipicolinate synthase (DHPS), or functional fragment thereof, having been provided with a mutation leading to the replacement of at least one single amino acid residue with a cysteine residue.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL 00/00942

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/82 C12N9/88 C12N5/04 A23K1/16 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A23K A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, EPO-Internal, MEDLINE, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SHAVER JONATHAN M ET AL: "Single-amino acid substitutions eliminate lysine inhibition of maize dihydrodipicolinate synthase." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 93, no. 5, 1996, pages 1962-1966, XP000918975 1996 ISSN: 0027-8424 cited in the application page 1963, left-hand column, paragraph 2	15, 17-19, 22-26
A	paragraph '0002!; figure 2	1-6,9-23

-/--

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search
23 July 2001

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2

INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL 00/00942

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GHISLAIN MARC ET AL: "A dinucleotide mutation in dihydrodipicolinate synthase of Nicotiana sylvestris leads to lysine overproduction." PLANT JOURNAL, vol. 8, no. 5, 1995, pages 733-743, XP000940840 ISSN: 0960-7412 cited in the application page 734, left-hand column, line 13	15, 17-19, 22-26
A	figures 4,6	1-6,9-23
A	BLICKLING STEFAN ET AL: "Feedback inhibition of dihydrodipicolinate synthase enzymes by L-lysine." BIOLOGICAL CHEMISTRY, vol. 378, no. 3-4, 1997, pages 207-210, XP000940920 ISSN: 1431-6730	1-26
Y	page 208, right-hand column, line 7; figure 3	7-26
Y	VAUTERIN MARC ET AL: "Isolation of a poplar and an Arabidopsis thaliana dihydrodipicolinate synthase cDNA clone." PLANT MOLECULAR BIOLOGY, vol. 25, no. 3, 1994, pages 545-550, XP000940934 ISSN: 0167-4412 cited in the application the whole document	7-26

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NL 00/00942

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-6 all completely and 9-26 all partially

   Nucleic acid molecules, vectors, host cells, selection methods, plants and food or feed relating to mutated DHPS genes. Also a method to increase lysine content of food or feed by transformation with mutant DHPS genes.

2. Claims: 7 and 8 all completely and 9-26 all partially

   As invention 1 but relating to wild type potato DHPS

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CORRECTED VERSION

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(51) International Patent Classification[7]: C12N 15/82, 9/88, 5/04, A23K 1/16, A01H 5/00

(21) International Application Number: PCT/NL00/00942

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(74) Agent: **PRINS, A., W.**; Vereenigde, Nieuwe Parklaan 97, NL-2587 BN The Hague (NL).

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(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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(54) Title: MODIFIED DHPS GENES

(57) Abstract: The invention relates to modifications of biosynthesis key enzymes leading to essential amino acid accumulation in cells. A recombinant or isolated nucleic acid, or functional fragment thereof, encoding an enzyme dihydrodipicolinate synthase (DHPS), or functional fragment thereof, having been provided with a mutation leading to the replacement of at least one single amino acid residue with a cysteine residue.

Title: Modified DHPS genes

The invention relates to modifications of biosynthesis key enzymes leading to essential amino acid accumulation in cells.

Human and monogastric animals cannot synthesise 10 out of 20 amino acids and therefore need to obtain these essential amino acids from their diet. The essential amino acids are lysine, leucine, isoleucine, valine, phenylalanine, methionine, threonine and tryptophan. Additionally, tyrosine and cysteine, although they are not strictly essential, must be considered as such, since they are synthesised only from essential amino acids: tyrosine from phenylalanine and cysteine from methionine. The diet (herein also called food respectively feed) of man and his livestock is largely based on plant material. Among the essential amino acids needed for animal and human nutrition some amino acids, such as lysine, are often only present in relatively low concentrations in crop plants. Therefore, synthetic amino acids are usually added as supplements to grain-based and other diets, in order to increase the nutritional value of the diet. Dietary proteins are not nutritionally equivalent, which correlates with the amino acid composition of the different proteins. Feeding a diet that provides an inadequate amount of one of the essential amino acids leads to negative nitrogen balance, since the normal catabolism of proteins continues, but new synthesis for replacement is limited by the relative lack of the essential amino acid. This occurs even when the total dietary intake of protein is apparently adequate. The extent to which a dietary protein can be used for the synthesis of tissue proteins is limited by the content of the essential amino acid that is present in the least amount relative to the requirement. This is the limiting amino acid of that protein.

Many crop plants contain especially low levels of lysine. Because of this, the lysine content of plants is an agronomically important trait and various attempts have been made in the past to increase the level of lysine in plants by classical breeding, mutant selection or by genetic modification.

Regulation of amino acid biosynthesis is mainly based on feedback inhibition of the key enzymes from the pathway by the amino acid end products. Problems

encountered by increasing the essential amino acid content in plants are due to the feedback inhibition mechanism.

The essential amino acid lysine is synthesised, together with threonine and methionine, from aspartate by a complex pathway which is similar for bacteria and higher plants (Figure 1). The aspartate family pathway has been characterised in detail in *Escherichia coli* by isolation of enzymes involved in the pathway, which were later also purified from higher plants (Bryan, J.K. (1980), The Biochemistry of Plants (ed. by B.J. Miflin) Vol. 5: 403-452, Academic Press, N.Y.; Umbarger, H.E. (1978) Ann. Rev. Biochem. 47: 533-606).

The rate of synthesis of the aspartate-family amino acids is regulated primarily by a complex process of feedback inhibition of the activity of some key enzymes in the pathway by the relevant amino acid end product. The first enzymatic activity in the pathway that is common to all of the aspartate-family amino acids, aspartate kinase (AK) activity, is feedback inhibited by both lysine and threonine. In addition, lysine also inhibits the activity of the enzyme dihydrodipicolinate synthase (DHPS), the first enzyme of the pathway after the branch point that leads to the synthesis of lysine. AK catalyses the phosphorylation of aspartate to form 3-aspartyl phosphate, with the accompanying hydrolysis of ATP. Both in *E. coli* and in plants several different AK isoenzymes have been identified which are differentially inhibited either by lysine or by threonine. The product of AK activity, 3-aspartyl phosphate, is in the next enzymatic step converted to 3-aspartic semialdehyde (3-ASA), which serves as a common substrate for the synthesis of both lysine and threonine. The enzyme dihydrodipicolinate synthase (DHPS) catalyses the first reaction that is unique to lysine biosynthesis, the condensation of 3-aspartate semialdehyde with pyruvate to form 2,3-dihydrodipicolinate. In *E. coli* this enzyme is encoded by the dapA locus and appears to consist of four identical subunits as a homotetramer (Shedlarski, J.G. and Gilvarg, C. (1970) J.Biol.Chem. 245: 1362-1373). The *E. coli* dapA gene has been cloned and sequenced (Richaud, F. et al. (1986) J.Bacteriol. 166: 297-300). In plants DHPS enzyme also appears to be a single enzyme comparable to the *E.coli* enzyme. All plant DHPSs purified sofar were shown to possess activity that is strongly inhibited by lysine alone. Among the major regulatory enzymes of the aspartate family pathway in plants, DHPS is the most sensitive to feedback inhibition by its

end product (I_{50} of DHPS for lysine ranges between 10 and 50 microM). DHPS is about 10-fold more sensitive to lysine inhibition than are plant lysine-sensitive AKs (I_{50} between 100 and 700 microM) and about 100-fold more sensitive to lysine inhibition than *E. coli* DHPS (I_{50} is about 1mM) (Yugari, Y. and Gilvarg, C. (1962) Biochem.Biophys.Acta 62: 612-614; Galili, G. (1995) The Plant Cell 7: 899-906).

Several lines of evidence have indicated that in plants DHPS is the major rate-limiting enzyme for lysine synthesis. Mutants of several plant species possessing feedback-insensitive AK isozyme were found to overproduce free threonine, but exhibited only a slight increase in the level of lysine (Bright,S.W.J. et al. (1982) Nature 299: 278-279; Cattoir-Reynaerts A. et al. (1983) Biochem. Physiol. Pflanzen 178: 81-90; Dotson, S.B. et al. (1990) Planta 182: 546-552; Frankard, V. et al. (1992) Plant Physiol. 99: 1285-1293). On the other hand, a feedback-insensitive DHPS mutant tobacco plant overproduced lysine (Negrutiu, I. et al. (1984) Theor.Appl.Genet. 6: 11-20; Shaver, J.M. et al. (1996) P.N.A.S. USA 93: 1962-1966). Similar results have been reported with transgenic plants expressing feedback-insensitive DHPS or AK from *E. coli* (Glassman, K.F. (1992) Biosynthesis and Mol. Regul. of Amino Acids in Plants (ed. by B.K. Singh et al.): 217-228; Perl, A. et al. (1992) Plant Mol.Biol. 19: 815-823; Shaul, O. and Galili, G. (1992a) Plant J. 2: 203-209; Shaul, O. and Galili, G. (1992b) Plant Physiol. 100: 1157-1163). Transgenic plants that expressed the *E. coli* DHPS overproduced lysine, while those that expressed the *E. coli* AK overproduced threonine and exhibited only a slight increase in the level of lysine.

European Patent Application No. 429458 discloses a method of increasing the level of free L-lysine in a plant comprising (a) introducing a foreign gene into the cells of a plant tissue source and (b) expressing the foreign gene in the cells where a first DNA sequence of the gene encodes dihydrodipicolinate synthase (DHPS) which is resistant to feedback inhibition by endogenously produced free L-lysine. The foreign gene may comprise a second DNA sequence attached to the 5' terminus of the first DNA sequence and which encodes a chloroplast transit peptide (CTP) which localises the DHPS in the chloroplast of the cells. The plants are said to produce elevated levels of free lysine. European Patent Application No. EP 93908395 describes two isolated DNA fragments comprising a fragment encoding AK insensitive to inhibition

by lysine and a second fragment encoding DHPS which is at least 20-fold less sensitive to inhibition by lysine than plant DHPS. It is claimed that the lysine-insensitive AK causes a higher than normal threonine production and that the DHPS causes a higher than normal lysine production in transformed plants.

The same results were found when feedback-insensitive bacterial DHPS and AK enzymes encoded by the *Corynebacterium* dapA gene and a mutant *E. coli* lysC gene, respectively, were expressed together in transgenic canola and soybean seeds. Several hundred-fold increases in free lysine in transgenic seed was observed, whereas the accumulation of excess threonine that was seen in transgenic seed expressing feedback-insensitive AK alone was prevented by the co-expression of DHPS (Falco,S.C. et al. (1995), Bio/Technology 13: 577-582). U.S. Patent Application No. 5773691 relates to four chimeric genes, a first encoding a bacterial lysine-insensitive aspartate kinase (AK), which is operably linked to a plant chloroplast transit sequence, a second encoding a bacterial lysine-insensitive dihydropicolinate synthase (DHPS), which is operably linked to a plant chloroplast transit sequence, a third encoding a lysine-rich protein, and a fourth encoding a plant lysine ketoglutarate reductase, all operably linked to plant specific regulatory sequences. The seeds of the transgenic plants are said to accumulate lysine or threonine to higher levels than untransformed plants.

The invention provides a recombinant or isolated nucleic acid, or functional fragment thereof, encoding an enzyme dihydrodipicolinate synthase (DHPS), or functional fragment thereof, having been provided with a mutation leading to the replacement of at least one single amino acid residue with a cysteine residue. The presence of an additional cysteine residue for example may result in the formation of alternative or additional sulphur bridges. As a result the overall structure and/or flexibilty of the enzyme will be affected, leading in the preferred cases to the loss of lysine feedback inhibition. In the present application a DHPS gene was used which was desensitised to lysine by at least one directed amino acid residue modification, preferably at the site of a in a wild-type enzyme conserved asparagine residue, and re-inserted into the cell of origin. In a preferred embodiment of the invention the replacement is located at or about the putative lysine binding site of DHPS, said site

in *Escherichia coli* located at around position 75-85, preferably at the site of a in a wild-type enzyme conserved asparagine residue.

The mutated gene is preferably put back in its original environment to which its expression, activity and interaction with other components is maximally adapted. Changing the endogenous enzyme by changing at least a single amino acid residue in the enzyme in its original host, such as a yeast, fungus, a plant or a bacterium, will also be far more acceptable to public and consumers than replacing the gene by a heterologous one.

In particular, the invention provides mutation of plant DHPS genes; genes encoding DHPS from various plant species have already been cloned, such as from *Nicotiana sylvestris* (Ghislain, M. et al. (1995) Plant J.8: 733-743), soybean (Silk, G.W. (1994) Plant Mol Biol 26: 989-993), wheat (Kaneko, T. et al. (1990) J. of Biol. Chem. 265: 17451-17455) and maize (Frisch, D.A. et al. (1991) Mol. Gen. Genet. 288: 287-293). Plant DHPS mutants with reduced sensitivity to lysine have been sought in order to use the mutated plant genes for increase of free lysine in plants. Direct selection of tobacco (*Nicotiana sylvestris*) UV-irradiated tissue cultures for selection to the lysine analog S-2-aminoethyl-L-cysteine (AEC) resulted in a dominant mutation that reduced lysine inhibition of DHPS and increased the concentration of free lysine in leaves and seed (Negrutiu, I. et al. (1984) Theor. Appl. Genet. 68: 11-20). However, no other plants with altered DHPS inhibition have been obtained by AEC selection. The tobacco mutated DHPS gene showed to contain a dinucleotide mutation located in a 10-amino acid-long region that presumably identifies the lysine-binding site of DHPS enzyme (Ghislain, M. et al. (1995) Plant J. 8: 733-743). The mutation resulted in the change of a conserved asparagine amino acid residue into a isoleucine residue. The change to obtain a di-nucleotide mutation needed for the conversion of asparagine into isoleucine by UV irradiation is extremely low, which could explain the fact that no other AEC resistant plants could be rescued.

Two other groups tried to mutate the conserved region involved in lysine binding of the plant DHPS enzymes in different ways. Shaver et al. ((1996) Proc. Natl. Acad. Sci. 93: 1962-1966) expressed the maize DHPS cDNA in an *E.coli dapA*⁻ auxotroph, treated the cells with the mutagens ethylmethanesulfonate (EMS) and selected the cells on the lysine analogue AEC. They found several single nucleotide changes

resulting in amino acid replacements causing nearly complete lack of lysine inhibition. However, no mutation was observed at the conserved asparagine residue which is changed in the potato DHPS enzyme as provided herein. Introduction of gene constructs containing these mutated maize DHPS genes into maize cell cultures resulted into an increase of four times the lysine content in transformed cells compared to wild type cells in the case of mutant DHPS166av (Bittel, D.C. et al. (1996) Theor. Appl. Genet. 92: 70-77). The use of this mutated maize DHPS gene is also mentioned in U.S. Patent Application No. 5545545.

The group of Silk and Matthews ((1997) Plant Mol Biol. 33: 931-933) used the information of the above mentioned mutations in tobacco and maize to change conserved amino acids in the soybean DHPS enzyme. The cloned soybean DHPS cDNA was site-directed mutated by PCR. One mutant contained a single amino acid substitution at codon 104 (conserved asparagine was changed into isoleucine at the same location in the tobacco DHPS mutant), in another mutant a single amino acid substitution was performed at position 112 (conserved alanine was changed into valine at the same location in the maize DHPS mutant). A third mutant contained both mutations. All mutants show the same lysine insensitivity when expressed in *E.coli dapA⁻* auxotroph cells. There are at present no data on expression of these mutant DHPS gene constructs in plant cells.

When the amino acid sequences of several bacterial and plant DHPS enzymes are compared, a number of conserved amino acids become clear (Shaver et al. ((1996) Proc. Natl. Acad. Sci. 93: 1962-1966). The invariant lysine residue at position 161 in E.coli is required for binding pyruvate. Furthermore, there are three amino acid residues very conserved in the 10 amino acid long stretch of the DHPS enzyme which is probably or putatively involved in the binding of lysine. The site comprises the conserved glycine at position 78 (position in E. coli), a conserved asparagine at position 80, and a conserved threonine at position 82. An amino acid residue which according to the invention is preferably replaced by cysteine is the conserved asparagine at position 80 (position 134 in potato DHPS and position 158 in corn). This same conserved amino acid residue was mutated in a tobacco DHPS mutant, however it was changed into an isoleucine in stead of a cysteine. The mutation of asparagine, or of an amino acid close to that asparagine, in cysteine is apparently

central to the increased lysine production of the transgenic organism, surprisingly, however, until now in none of cloned wild type and mutant bacterial, fungal or plant DHPS genes a cysteine residue is found at or around the (in wild type enzymes) conserved asparagine position.

The invention also provides a method for selecting a transformed host cell comprising a recombinant nucleic acid, or functional fragment thereof, encoding an enzyme dihydrodipicolinate synthase (DHPS), or functional fragment thereof, comprising culturing host cells in the presence of S-(2-aminoethyl)-L-cysteine and selecting a desired host cell for relative feedback insensitivity to S-(2-aminoethyl)-L-cysteine. Due to the combination of a randomised site-directed mutation method and selection for feedback insensitivity the invention provides an extremely insensitive DHPS mutant which, when for example expressed in potato plants, resulted in an accumulation of the free lysine content of more than 30% of the total free amino acid level in tubers. Increase of the lysine levels in potato is far more difficult than in, for instance tobacco, as can be seen by the experiments from Perl et al ((1993) Plant Mol Biol 19: 815-823) where expression of the bacterial DHPS (DapA) gene in potato resulted in far less lysine accumulation in potato than compared to expression of this gene in tobacco.

The invention also provides recombinant or isolated nucleic acid, or functional fragment thereof, encoding potato DHPS, or functional fragment thereof, preferably wherein said nucleic acid is at least 60%, preferably at least 75%, most preferably at least 90% homologous to a nucleic acid as shown in figure 2A. We mutagenised by PCR very specifically the conserved aspargine residue at position 134 in said potato DHPS enzyme into all other possible amino acid residues. The pool of mutant DHPS clones obtained in this way, containing all possible amino acid residues at position 134 was subsequently expressed in *E.coli dapA⁻* auxotroph cells and selected for lysine insensitivity with the lysine analogue AEC. The conserved amino acid residue that had to be mutated was selected by us, but the change to another amino acid residue was driven by the selection in *E.coli dapA⁻* cells.

The invention also provides a promotor or functional fragment thereof to drive expression of a DHPS gene as provided by the invention. Suitable promotors, such as plant-specific promotors are known in the art. In particular, the invention provides a

nucleic acid according to the invention further comprising a tissue-specific promotor or functional fragment thereof. A preferred promoter to drive expression of the DHPS enzyme according to the invention is the tuber-specific granule bound starch synthase (GBSS) promoter (Visser, R.G., Plant. Mol. Biol. 17:691-699, 1991). Other tuber-specific promoters can be used to drive DHPS expression like class I patatin promoter from *Solanum tuberosum* (Mignery, C.A. et al. (1988) Gene 62: 27-44; Wenzler, H.C. et al. (1989) Plant Mol.Biol. 12: 41-50), AGPase promoter (Muller-Rober, B., et al., Plant Cell 6:601-612, 1994), proteinase inhibitor II promoter from potato (Keil, M. et al. (1989) EMBO J. 8: 1323-1330), or the cathepsin D inhibitor promoter from potato (Herbers, K. et al. (1994) Plant Mol. Biol. 26: 73-83). Also other tissue-specific promoters can be used like the fruit-specific promoter from tomato polygalacturonidase gene (Grierson, D. et al. (1986) Nucl.Acids Res. 14: 8595-8603), or the seed-specific phaseoline promoter from bean (Sengupta-Gopalan, C. (1985) Proc.Natl.Acad.Sci.USA 82: 3320-3324). Other promoters that can be used are inducible promoters, like the light inducible promoter derived from the pea rbcS gene (Coruzzi G. et al. (1984) EMBO J. 3: 1671-1679) and the actine promoter from rice (McElroy, D. et al. (1990) The Plant Cell 2: 163-171).

The promoter is in general to be found in the 5' region of each of the gene. Since the organelle in which lysine biosynthesis takes place in higher plants is the plastid, the gene construct comprises also a DNA sequence coding for a transit peptide which is involved in the translocation of the protein from the cytosol into the plastids (Van den Broeck, G. et al. (1985) Nature 313: 358-363; Schreier, P.H. et al. (1985) EMBO J. 4: 25-32). The chloroplast targeting signal can either naturally be present in a DHPS gene originating from a plant or has to be added when the DHPS gene originates from bacterial, fungal or algal organisms. This DNA sequence encoding a chloroplast transit peptide fused to a DNA sequence coding for DHPS, will on expression produce a fused DHPS/transit peptide chimeric protein in the cytoplasm of the transformed plant cell, which will be transported to the plastids, where increased production of lysine is thereby obtained.

In a particular embodiment of the invention, the 3' end of the DNA sequence coding for the transit peptide is fused to the DNA sequence encoding DHPS, which is fused to a transcription termination DNA signal. This termination signal comprises a

3' transcription termination and a mRNA polyadenylation signal. Termination signals present at the 3' flanking region of any cloned gene can be used, e.g. from the pea rbcS gene, the bean phaseoline gene, or the nopaline synthase gene derived from the Ti plasmid of *Agrobacterium tumefaciens*. A preferred terminator sequence originates from the 3' flanking region of the octopine synthase gene from the Ti plasmid of *Agrobacterium tumefaciens* (Figure 3; Greve, H.D. et al. (1983) J.Mol.Appl.Genet. 1: 499-511).

The invention also provides a vector comprising a nucleic acid according to the invention. For example, an expression vector can be of the class of high copy number pUC or pBR322 derived plasmids, which can be used for direct transformation methods, like electroporation, particle bombardment or poly-ethylene-glycol mediated transformation procedures. Alternatively, expression constructs are included on a Ti plasmid derived, so-called binary vector, like pBINPLUS (Van Engelen, F.A. et al. (1995) Transgenic Research 4: 288-290). The Ti plasmids can be propagated in *Agrobacterium tumefaciens*, from which the inserted DNA fragments between the left and right border are transferred to the plant cell.

The expression vector in which the gene construct as provided by the invention, such as a chimeric gene construct, is cloned, is introduced into host cells such as fungal, bacterial, algal or plant cells. The introduction is realised using any kind of transformation protocol capable of transferring DNA; for plants several protocols have been developed for to either monocotyledonous or dicotyledonous plant cells. For example: transformation of plant cells by direct DNA transfer via electroporation (Dekeyser, R.A. et al. (1990) The Plant Cell 2: 591-602), via PEG precipitation (Hayashimoto, A. et al. (1990) Plant Physiol. 93: 857-863) or via particle bombardment (Gordon-Kann, W.J. et al. (1990) The Plant Cell 2: 603-618), and DNA transfer to plant host cells via infection with Agrobacterium. A preferred method is via infection of plant cells with *Agrobacterium tumefaciens* (Horsch, R.B. et al. (1985) Science 227: 1229-1231; Visser, R.G.F. (1991) Plant Tissue Culture Manual B5 (ed. by K. Lindsey): 1-9, Kluwer Acad. Publishers, The Netherlands). The methodology used here for in the detailed description for potato or grass is also provided to improve many other crop plants such as for example transgenic sugar beet, carrot, cassave, canola, alfalfa, legumes, and gramineae species like rice, maize, wheat, sorghum,

barley and grasses like *Lolium perenne*, in a preferred embodiment of the invention, said plant comprises a tuber, such as a potato, from which lysine rich plant material can be obtained by tissue specific expression as provided herein. Other specific tissues wherein the DHPS-gene preferentially can be expressed comprise roots and seeds.

Transformed plants are for example selected by resistance to kanamycin or other antibiotics like hygromycin, or herbicides like bialaphos or phosphinotricin.

Alternatively, desired transgenic plants or transgenic host cells (from which plants can be obtained) with a high lysine content are selected by cultivation in the presence of a lysine analogue, such as S-(2-aminoethyl)-L-cysteine (AEC), without selection on resistance to antibiotics or herbicides, as described above where E. coli host cells were selected. In a preferred example, the tissue-specific promoter that is used to drive DHPS expression is activated during the AEC selection. For example, the tuber-specific granule bound starch synthase (GBSS) promoter is induced by high levels of sucrose [Visser, R.G.F. et al. (1991) Plant Mol. Biol. 17:691-699), as is the AGPase promoter (Muller-Rober, B. et al. (1990) Mol. Gen. Genet. 224: 136-146) and the class I patatine promoter (Wenzler, H.C. et al. (1989) Plant Mol. Biol. 12: 41-50). Other tissue-specific promoters like the seed-specific phaseoline promoter from bean and the zein promoter from maize are induced during AEC selection by addition of ABA (Muller, M. et al. (1997) Plant J. 12 (2): 281; Bustos, M.M. et al. (1998) Plant Mol. Biol. 37(20): 265). No marker genes except for the DHPS gene thus have to be introduced.

The invention furthermore provides a method to obtain plant material with relative high lysine content comprising harvesting at least a part of a plant according to the invention. Harvesting crop plants and obtain specific plant material, such as seeds, leaves, stems, roots, tubers or fruits, is a skill known in the art, allowing to obtain plant material from a plant according to the invention. The invention thus provides a method to increase lysine content of food or feed with relative low lysine content comprising adding plant material according to the invention to said food or feed. Lysine rich food or feed is also provided, and last but not least, it is in itself possible to use the lysine-enriched plant material according to the invention, such as

lysine enriched potatoes, grasses, seeds or fruits as food or feed. The invention is further explained in the detailed description without limiting the invention.

Detailed description.

Example 1. Cloning of the potato DHPS cDNA

DNA isolation, subcloning, restriction analysis and DNA sequence analysis is performed using standard methods (Sambrook, J. et al. (1989) Molecular Cloning. A laboratory manual, Cold Spring Harbor Laboratory Press; Ausubel, F.M. et al. (1994) Current protocols in molecular biology, John Wiley & Sons).

For the isolation of the *Solanum tuberosum* cv Kardal gene encoding dihydrodipicolinate synthase (DHPS) a cDNA library of mRNA prepared from young tubers was constructed in the vector λTriplEx (Clotech). A specific 600 basepairs DHPS DNA fragment for screening the cDNA library via heterologous hybridisation was cloned by the reverse transcriptase polymerase chain reaction (RT-PCR) on total RNA isolated from *Arabidopsis thaliana* and subsequently ligated into vector pMOSBlue (Amersham), resulting in pAAP15. To this purpose synthetic oligonucleotides based on the sequence of the *Arabidopsis thaliana* DHPS gene were used (Vauterin & Jacobs, 1994).

A single plaque from the cDNA hybridising to pAAP15 was selected and the 1190 basepairs DNA insert completely sequenced (Figure 2A). It was concluded to encode a dihydridipicolinate synthase. The DNA fragment encompassing the complete coding sequence was cloned in the cloning vector pBluescript SK(+) (Stratagene) via a PCR-based strategy to enable the expression in *Escherichia coli* of a β-galactosidase-DHPS fusion protein under the control of a *lac* promoter. The resulting plasmid was designated pAAP57. This gene construct was able to complement *E. coli* AT997 (Yeh et al., 1988), a DHPS deficient strain, allowing it to grow on minimal medium in the absence of DL-α,ε-diaminopimelic acid.

Example 2. Mutagenesis to create a lysine insensitive DHPS gene

In order to create a feedback insensitive DHPS, nucleotides in pAAP57 corresponding to the evolutionary conserved amino acid residue 134 (asparagine) were changed at random via a PCR-based approach (QuickChange site directed mutagenesis, Stratagen), resulting in a population of plasmids encoding DHPS enzymes with different amino acids residues at position 134. Following transformation of *E. coli* AT997 with this plasmid population, selection for feedback insensitivity was done in the presence of 1 mM of the lysine analogue S-(2-aminoethyl)-L-cysteine (AEC). Several AEC resistant colonies were picked and the DHPS coding region of their plasmid directing AEC resistance sequenced (Figure 2B). The change of AAC into either TGT or TGC resulted in the corresponding change of the asparagine residue at DHPS position 134 into a cysteine residue. The mutant DHPS encoding DNA fragment (designated DHPS-134nc1) was used for the expression in potato plants.

Example 3. Chimeric gene construct with the mutant potato DHPS gene

The chimeric gene containing the mutant DHPS gene was constructed by subcloning DHPS cDNA from the pTriplex vector (pAAP42) first in pCR-Script SK(+) (pAAP55) and from this vector as a XbaI–Eco RI fragment in the pBluescript SK vector digested with XbaI–EcoR (pAAP57). With this clone the mutagenesis was performed, resulting in clone pAAP57-134nc1. At the 5'end the mutated DHPS cDNA was fused to a HindIII–SalI fragment of the 800 bp long GBSS promoter fragment (Visser et al. ibid). Downstream of the mutant DHPS sequence the termination signal of the nopaline synthase gene from *Agrobacterium tumefaciens* was inserted (Greve, H.D. et al. (1983) J.Mol.Appl.Genet. 1: 499-511) as an SstI–EcoRI fragment. The complete chimeric gene was subcloned into the HindII–EcoRI sites of pBINPLUS (Van Engelen, F.A. et al. (1995) Transgenic Research 4: 288-290) (pAAP105, Figure 3).

Example 4. Introduction of the chimeric gene into potato

4.1 Transformation of potato plants

The binary vector pAAP105 was used for freeze-thaw transformation of *Agrobacterium tumefaciens* strain AGLO (Höfgen, R. and Willmitzer, L. (1988) Nucl.Acids Res. 16: 9877). Transformed AGLO was subsequently used for inoculation of potato (*Solanum tuberosum*, variety Kardal) stem explants as described by Visser (Visser, R.G.F. (1991) Plant Tissue Culture Manual B5 (ed. by K. Lindsey): 1-9, Kluwer Acad. Publishers, The Netherlands). After shoot and root regeneration on kanamycin-containing media plants were put in soil and transferred to the greenhouse. Plants regenerated (on kanamycin-free media) from stem explants treated with the Agrobacterium strain AGLO lacking a binary vector served as a control.

4.2 Selection of transformed plant cells on AEC

In stead of selection of transformed plant cells on kanamycine, as described by Visser (Visser, R.G.F.(1991) in : K. Lindsey (ed) Plant Tissue Culture Manual B5: 1-9, Kluwer Acad Publishers) mentioned above (4.1), the transformed plant cells containing a mutant DHPS gene can also be selected on the lysine analogue AEC. After inoculation of for example potato stem explants with the transformed (AGI 0) strain, explants are in general first cultured on medium without AEC. After a short period, for example 12-20, such as 16, days they are transferred to medium with 0.1 mM AEC and a sufficient concentration (e.g. 5%) sucrose. When the first primordia are visible the explants are transferred to 0.025 mM AEC and sucrose. Root induction is performed in the absence of AEC/sucrose-selection.

4.3 In vitro tuber formation

In order to induce in vitro tuberisation, nodal cuttings (about 4-5 cm long) of transformed potato plantlets are placed vertically in solid Murashige and Skoog

medium (Murashige, T. and Skoog, F. (1962) Physiol.Plant. 15: 473-497) supplemented with 10% (w/v) sucrose, 5 TM BAP. The cultures are maintained at 19°C in the dark. After 14 days microtubers of 4 mm in diameter are harvested and analysed for free lysine and threonine content, and for AK and DHPS activity. The protein content and the protein composition are analysed as described below.

4.4. Selection of transformed shoots with high DHPS* expression on aminoethylcysteine (AEC)

In stead of selection on kanamycin after transformation with the pAAP105 construct by Agrobacterium inoculation (see example 4.1), the transformed shoots can be selected by rooting on a medium containing a lysine analogue. The mutant DHPS gene can be used as a selection marker because plant cells with a high content of lysine can grow on toxic lysine analogues, such as aminoethylcysteine (AEC). Due to the feedback-insensitivity and resulting high endogenous lysine levels, the toxic analogue is diluted out and cannot bind to the lysine binding site in the mutant enzyme.

After transformation of potato stem explants (see 4.1) with the pAAP105 containing Agrobacterium strain, the explants were put on shoot regeneration medium without kanamycin. When shoots appeared they were either put directly on rooting medium containing 0.06 mM AEC with 50 g/l sucrose (for induction of the GBSS promoter), or were rooted first on rooting medium without AEC and afterwards were rooted again on medium containing 0.06 mM AEC and 50 g/l sucrose. Shoots that were able to root on this concentration AEC were cut again for rooting on medium containing 0.1 mM AEC and 50 g/l sucrose. The criteria for rooting resistance on 0.06 and 0.1 mM AEC are the following:

- roots should appear at the cutting edge of the shoot
- cutting edge should not be yellow-brownish and rotten
- root tips should be light coloured

The transformation frequency leading to transgenic plants with eventually high lysine levels in the tubers is about 0.5%.

Example 5. DHPS modification of perennial ryegrass (*Lolium perenne* L.)

Plant materials

Embryogenic suspension cultures from different *Lolium perenne* cultivars, a.o. the cultivars Moronda and Aurora, are initiated directly from mature, seed-derived embryos or from embryogenic callus cultures obtained from immature inflorescence segments, essentially as described by Creemers-Molenaar et al., *Plant Science* 63:167-176 (1989). For the direct approach, seeds are sterilized in 10% hypochlorite, rinsed and soaked for two days in sterile tap water and sterilized for a second time. After rinsing thoroughly, mature embryos are dissected from 40 seeds, chopped and transferred to 5 ml of MS10 medium (i.e. Murashige and Skoog basal salts and vitamins supplemented with 10 mg/l 2,4-D and 3% sucrose at pH 5.8) in a 60 ml plastic specimen container (Thovadex). This in several replicates. Embryogenic callus is induced on immature inflorescence segments of greenhouse grown plants after sterilization with 5% hypochlorite and rinsing with sterile water. The basal parts of the inflorescences are cut into 2 segments of 1-2 mm long and placed on MSt5 medium (i.e. MS basal salts and vitamins supplemented with 0.4 mg/l (=extra) thiamin-HCl, 5 mg/l 2,4-D and 3% sucrose) solidified with 0.8% Daichin agar. Culture is in the dark at 25 °C. After 4-8 weeks compact, embryogenic callus is excised from the explants of 2-5 plants (i.e. genotypes). The calli are mixed, chopped with a scalpel and transferred in 0.1 g FW aliquots to 5 ml of MS10 in specimen containers. From this point on, the cultures of both origins are treated identically. The cultures are incubated on a rotary shaker (140 revs./min.) in continuous indirect light (200-400 lux) at 25 °C.

After 10 days the medium is replaced with MS5 (= as MS10 but with 5 mg/l 2,4-D). Once a week 2 ml of fresh medium is added to the cultures until a final volume of 15 ml is reached. Subsequently, the cultures are transferred to new 190 ml transparent, polystyrene containers (Greiner) and 5 ml fresh medium is added. For further experimentation well-proliferating, finely-dispersed cultures are selected and maintained by weekly subculturing 2.5 g FW material in 20 ml fresh MS5. Incubation is in the dark under continuous shaking at 120 revs./min. at 25 °C.

The regeneration potential of the cultures is determined by placing 0.5-1.0 g FW material on solid MS0 (0 mg/l 2,4-D; 0.8% agar). After culturing in the dark at 25 °C for the first 2 weeks the calli are placed in dimmed light (500-1000 lux) for 16 hours/day for the next 2 weeks. Finally after transfer to fresh MS0, they are placed in 4000 lux, 16 hours/day and the number of calli producing shoots is scored after 4 weeks.

Transformation

Three days after subculture, 0.25 g FW callus material consisting of cells in log-phase, is evenly dispersed onto the surface of a 42 mm diameter Whatman filter disc (Schleicher & Schuell #604). Subsequently, the filters are moistened by the addition of 0.5 ml fresh culture medium and they are placed onto culture medium solidified with 0.2% gelrite and left overnight at 25 °C in the dark. The next day the filters carrying the perennial ryegrass suspension material are used for biolistic gene transfer using the PDS1000-He particle gun (BioRad). 0.375 mg Gold particles with an average diameter of 1 μm were coated with 0.625 μg DNA of plasmid pAAP205, which is a derivative of the plasmid pAAP105 mentioned in Example 3 lacking T-DNA borders and with the *npt*II gene replaced by the *hpt* gene for selection of transgenic perennial ryegrass cells. For the coating, 50 μl (= 3 mg) of washed particles are suspended thoroughly by vortexing. Subsequently, 5 μl plasmid DNA (concentration 1 μg/μl) and 50 μl 2.5M $CaCl_2$ are added and vortexed for 10 seconds. Then, 20 μl 0.1M free-base spermidine is added and mixed by vortexing for 2 seconds. The mixture is centrifuged for 5 seconds and the supernatant is removed, after which 250 μl ethanol 96% is added followed by vortexing for 1 minute. After washing with ethanol once, the particles now coated with DNA are resuspended in 60 μl ethanol 96% and kept on ice until use. Bombardment can be performed at pressures ranging from 1100 psi to 2200 psi, but in this example particularly a pressure of 1800 psi is used while the dish containing the filters is placed at a distance of 9 cm. After biolistics the filters are incubated on the culture medium in the dark at 25 °C for 24 hours before they are transferred to selection medium. For selection, the filters are first placed on culture medium MSt5 solidified with 0.2% gelrite containing 80 mg/l hygromycin; after 1 week the filters are transferred to selection medium containing 150 mg/l hygromycin. So far, this is essentially as described earlier (Van der Maas et

al., *Plant Mol. Biol.* 24:401-405 [1994]). Actively growing calli are individually transferred to fresh selection medium (150 Hyg.) after 4 weeks. Following this second round of selection surviving calli are placed on regeneration medium supplemented with 50 mg/l hygromycin. Transgenic perennial ryegrass plants are obtained and collected after 8 weeks. They are maintained in tubes containing half strength MS0 (Creemers-Molenaar et al., *Plant Science* 57: 165-172 [1988]) and subsequently characterized molecularly by PCR and Southern hybridization analysis and biochemically by amino acid, enzyme activity and protein analysis as described in Example 6 - 9 to confirm presence and expression of the newly introduced gene constructs.

Example 6. Analysis of free amino acid content in transgenic plants

Tissue (0.5-1.0 gram) was homogenised with mortar and pestle in 2 ml 50 mM Pi-buffer (pH 7.0) containing 1 mM dithiothreitol. Nor-leucine is added as an internal standard. Free amino acids were partly purified by extraction with 5 ml of a water:chloroform:methanol mixture (3:5:12). Water phase was collected and the remaining re-extracted twice. After concentration by lyophilisation to 3 ml, a 20 μl sample was analysed by HPLC using a cation-exchange column with post-column ninhydrine derivatisation of the amino acids detected at 570 and 440 nm (BIOCHROM 20, Amersham Pharmacia biotech). Figure 4 shows the lysine levels as the percentage of the total amino acids in tubers in 27 untransformed potato plants and in 30 potato plants containing the mutated DHPS gene construct pAAP105. The lysine levels increase from 2-2.5% in the control wild type tubers to a maximum percentage of 30 in the transformed tubers, resulting in lysine being a 'bulk' amino acid in stead of a 'low level' essential amino acid .

Example 7. Analysis of DHPS enzyme activity in transgenic plants

Microtubers or mature tubers were homogenised with mortar and pestle in an equal volume of cold 100mM Tris-HCl pH 7.5 containing 2 mM EDTA, 1.4% sodium ascorbate, 1mM phenylmethylsulphonilfluoride and 0.5 Tg/ml leupeptin. Following 5 min. centrifugation (16,000 g at 4^0C) the supernatant is collected. DHPS activity was measured using the O-aminobenzaldehyde (O-ABA) method of Yugari and Gilvarg (Yugari, Y. and Gilvarg, C. (1965) J.Biol.Chem. 240: 4710-4716). As expected, AEC sensitivity of transformed was reduced, when comparing the wild type and mutant DHPS activity in respectively control (untransformed) and transformed potato tubers, the latter showed DHPS acitivity even under increased levels of AEC.

Example 8. DNA analysis of transgenic plants

Genomic DNA was isolated from potato tubers of plants grown in the green house. Lyopholised tissue was ground in liquid nitrogen and added to 15 ml extraction buffer (100mM Tris.HCl, pH 8, 500mM NaCl, 50mM EDTA and 10mM ß-mercaptoethanol) and 2 ml 10% SDS. After incubation at 65°C for 20 min, 5 ml of 5M KAc were added and incubated on ice for 15 min. After centrifugation, filtration of the supernatant, and precipitation with 15 ml iso-propanol, the pellet was resuspended in 400 µl TE and treated with 1 µg RNAse. The DNA was subsequently purified with CTAB (N-Cetyl-NNN tri-methylammonium bromide) extraction by adding 400 µl of CTAB buffer (200mM Tris-HCl pH 7.5, 50mM EDTA, 2mM NaCl and 2% CTAB) and incubating at 65 0C for 15 min. After extraction with 800 µl chloroform/isoamyl alcohol 24:1, the DNA was precipitated with 800 µl iso-propanol. The pellet was washed with 70% ethanol and resuspended in TE. DNA. After digestion with restriction enzymes, the DNA was size-fractionated on an agarose gel and blotted onto Hybond-N^+ (Amersham). Random-primer-labeled probe was derived from the DHPS cDNA (from position 337 to 811, Figure 2). Filters were hybridised at 65 ^{0}C in 10 % dextran sulphate, 1% SDS and 1M NaCL for 16 to 20 hr. and washed subsequently at 55 ^{0}C and 60 ^{0}C with 2x SSC, 0.1 % SDS, and at 65 ^{0}C with 0.1x SSC, 0.1% SDS.

Example 9. Expression analysis of transgenic plants on RNA level

Total RNA was isolated from potato tubers grown in the greenhouse according to the protocol described by Ausubel et al. (1994). RNA was size-fractionated on formaldehyde agarose gels and blotted onto Hybond-N^+ (Amersham). Blots were briefly stained to ensure that equal amounts of RNA were present. Random-primer-labeled probe was derived from the DHPS cDNA (from position 337 to 811, Figure 2). Filters were hybridised at 65 ^{0}C in 10 % dextran sulphate, 1% SDS and 1M NaCL for 16 to 20 hr. and washed subsequently at 55 ^{0}C and 60 ^{0}C with 2x SSC, 0.1 % SDS, and at 65 ^{0}C with 0.1x SSC, 0.1% SDS.

Figure legends

fig.1:

A diagram of the aspartate family biosynthetic pathway. Only the major key enzymes are indicated. Curved arrows represent feedback inhibition by the end product amino acids DHPS, dihydrodipicolinate synthase; HSD, homoserine dehydrogenasse; TDH, theonine dehydratase.

fig.2:

(A) Nucleic acid fragment encoding a DHPS isolated from potato. The derived amino acid sequence is presented using the 1-letter code.

(B) Nucleic acid sequence and derived amino acid sequence of a lysine binding domain of the mutated DHPS (DHPS-134nc.)

fig.3:

Schematic representation of the T-DNA region of plasmid pAAP105, encompassing a mutated dihydrodipicolinate coding region (DHPS) under control of the granule bound starch synthase promoter (P-GBSS) and as selection maker the neomycinephosphotransferase 11 coding region (NPT11) under control of the nopaline synthase promoter(P-NOS);I-NOS nopaline synthase transcription terminator.

fig.4:

Free lysine concentration(micromol/g fresh weight) of tubers from tubers derived from different, independent transformant or control plants. Plants were grown in pots in the green house until maturity. After harvesting tubers were dried for one week before analysis. About 10 gram of tuber material was ground in 50 ml of a 1 mM dithiothreitol (DTT) solution. Two ml of the resulting suspension was extracted with a menthanol: water: chloroform mixture(12:3:5). After concentration to 0.5 ml, 25µl is used for analysis on a Biochrom 20(Amersham-Phamacia) lithium-based ionexchange amino acid analysis system. Samples were spiked with L-Norleucine before grinding, to allow corrections.

fig. 5.

Southern blot DNA analysis of several transformed potato plants.

Genomic DNA was isolated from several independent transformed potato plants and untransformed plants, and digested with either HincII or with a combination of HincII and BamHI. After separation and blotting, the DNA fragments were hybridised with a radio-labelled DHPS probe. The untransformed control plants (C) show only hybridisation of the endogenous DHPS fragments. On top of those bands the transformed plants show one (lane 7) or more extra hybridising bands (e.g. lane 5) revealing the insertion of one or more copies of the GBSS-DHPS* construct. Abbreviations: C, control plants; M, molecular size marker.

Fig. 6.

Northern blot RNA analysis of the tubers of several transformed potato plants.

Total RNA was isolated from tubers of several transformed plants (lanes 1-15) and from control untransformed plant (lane C). RNA was-separated by gel-electrophoresis and blotted on nylon. The blot was hybridised with a radio-labelled DHPS DNA probe. Control RNA from an untransformed plant shows hybridisation of the endogenous DHPS RNA (lane C). RNA from several transformed plants show hybridisation which is equal to the background DHPS hybridisation (e.g. lanes 4 and 9). Most of the transformed plants show a strong hybridisation to the expressed exogenous DHPS* RNA (e.g. lanes 10-12).

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Claims

1. A recombinant or isolated nucleic acid, or functional fragment thereof, encoding an enzyme dihydrodipicolinate synthase (DHPS), or functional fragment thereof, having been provided with a mutation leading to the replacement of at least one single amino acid residue with a cysteine residue.

2. A nucleic acid according to claim 1 wherein said amino acid replacement is located at or about the putative lysine binding site of DHPS, said site in *Escherichia coli* located at around position 75-85.

3. A nucleic acid according to claim 1 or 2 wherein said single amino acid residue comprises an asparagine residue.

4. A nucleic acid according to anyone of claims 1 to 3 wherein in potato DHPS said asparagine residue is located at position 134.

5. A nucleic acid according to anyone of claims 1 to 4 wherein said DHPS is of bacterial, fungal, algal or plant origin.

6. A nucleic acid according to claim 5 wherein said plant is a potato.

7. A recombinant or isolated nucleic acid, or functional fragment thereof, encoding potato DHPS, or functional fragment thereof.

8. A nucleic acid according to claim 7 wherein said nucleic acid is at least 90% homologous to a nucleic acid as shown in figure 2A.

9. A nucleic acid according to anyone of claims 1 to 8 further comprising a tissue-specific promotor or functional fragment thereof.

10. A nucleic acid according to claim 9 wherein said promotor is derived from the tuber-specific granule bound starch synthase (GBSS) promotor.

11. A vector comprising a nucleic acid according to anyone of claims 1 to 10.

12. A host cell comprising a nucleic acid according to anyone of claims 1 to 10 or a vector according to claim 11.

13. A host cell according to claim 12 comprising a plant cell.

14. A host cell according to claim 13 wherein said plant cell is derived from a potato.

15. A method for selecting a transformed host cell comprising a recombinant nucleic acid, or functional fragment thereof, encoding an enzyme dihydrodipicolinate

synthase (DHPS), or functional fragment thereof, comprising culturing host cells in the presence of S-(2-aminoethyl)-L-cysteine and selecting a desired host cell for relative feedback insensitivity to S-(2-aminoethyl)-L-cysteine.

16. A method according to claim 15 wherein said nucleic acid comprises a nucleic acid according to anyone of claims 1 to 10.

17. A host cell obtainable with a method according to claim 15 or 16.

18. A plant comprising a host cell according to claim 14 or 17.

19. A plant according to claim 18 comprising a crop plant.

20. A plant according to claim 18 or 19 comprising a tuber.

21. A tuber derived from a plant according to claim 20.

22. A method to obtain plant material with relative high lysine content comprising harvesting at least a part of a plant according to claim 18.

23. Plant material obtainable by a method according to claim 22.

24. A method to increase lysine content of food or feed with relative low lysine content comprising adding plant material according to claim 23 to said food or feed.

25. Food or feed obtainable by a method according to claim 24.

26. Food or feed comprising plant material according to claim 24.

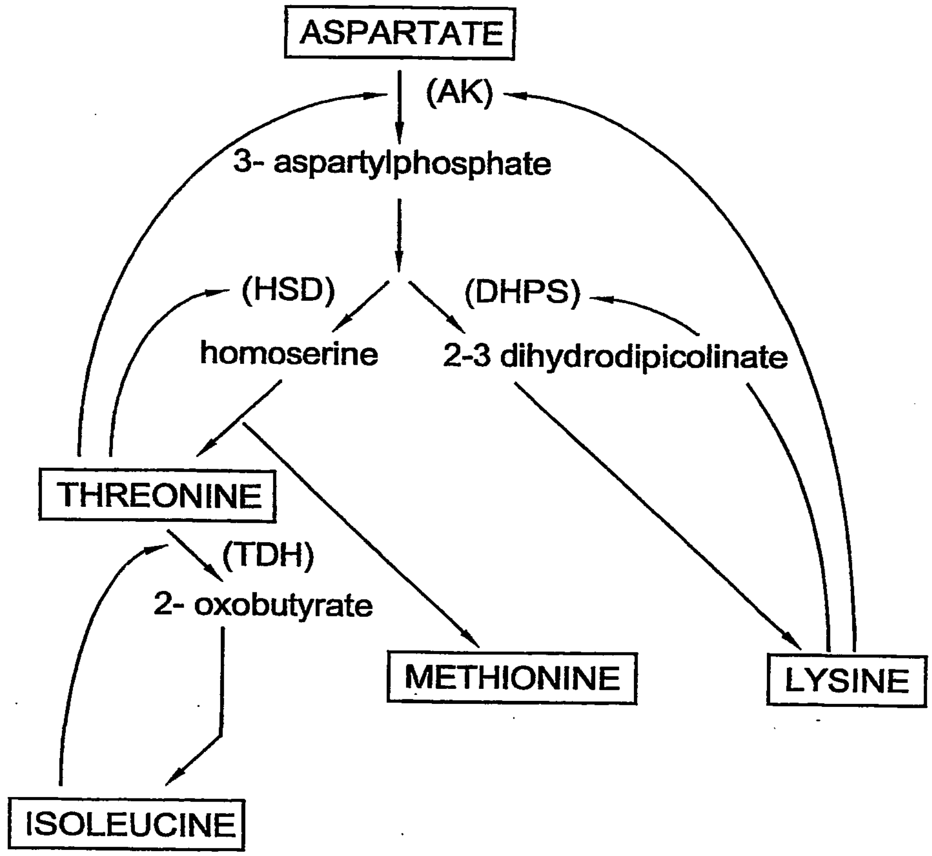
ASPARTATE
(AK)
3- aspartylphosphate
(HSD)
(DHPS)
homoserine
2-3 dihydrodipicolinate
THREONINE
(TDH)
2- oxobutyrate
METHIONINE
LYSINE
ISOLEUCINE

Fig. 1

2/6

Fig. 2A

```
CCGATAGGGA GAAATGTCAT CCTCAATTAC AAATTGCTGC CATTTCTATG    50
            M  S       S  S  I  T     N  C  C    H  F  Y

TTGAACGTGC CAGAACAAAC AGGAGAACTG CAACATGGAA GTCACCAAGA   100
V  E  R  A    R  T  N    R  R  T    A  T  W  K    S  P  R

GCAGCAGTAA TTCCATGTAT CCACCTCCCG ATGCGCAGTA ATGAAGTTAA   150
 A  A  V    I  P  C  I    H  L  P    M  R  S    N  E  V  K

AAATAGGACA TTTGCTGAGG ACATAAAAAA ACTTCGATTG ATTACTGCAA   200
 N  R  T     F  A  E    D  I  K  K    L  R  L    I  T  A

TTAAAACCCC ATATCTACCA GATGGAAGAT TCGATCTTGA GGCTTATGAT   250
I  K  T  P    Y  L  P    D  G  R    F  D  L  E    A  Y  D

GCCTTAGTAA ATTTGCAAAT TGAAAATGGT GTCGAGGGTG TGATTGTTGG   300
 A  L  V    N  L  Q  I    E  N  G    V  E  G    V  I  V  G

TGGCACTACA GGTGAAGGTC AATTGATGAG CTGGGATGAA CACATCATGC   350
 G  T  T     G  E  G    Q  L  M  S    W  D  E    H  I  M

TCATTGGTCA TACCATCAAT TGTTTTGGTG GATCAATTAA AGTCATTGGA   400
L  I  G  H    T  I  N    C  F  G    G  S  I  K    V  I  G

AACACAGGAA GCAACTCCAC GAGGGAAGCA ATCCATGCGA CAGAACAAGG   450
 N  T  G    S  N  S  T    R  E  A    I  H  A    T  E  Q  G

ATTTGCTGTA GGTATGCATG CAGCTCTTCA CATTAATCCT TACTATGGGA   500
  F  A  V    G  M  H    A  A  L  H    I  N  P    Y  Y  G

AGACCTCCAT AGAGGGTTTG ATTTCTCACT TTGACAGTGT GCTCCCTATG   550
K  T  S  I    E  G  L    I  S  H    F  D  S  V    L  P  M

GGCCCTACTA TCATTTACAA TGTGCCATCA CGAACTGGTC AAGACATTCC   600
 G  P  T    I  I  Y  N    V  P  S    R  T  G    Q  D  I  P

CCCTCATGTA ATCCAGACAT TAGCAAAGAG CCCTAACCTT GCTGGTGTCA   650
  P  H  V    I  Q  T    L  A  K  S    P  N  L    A  G  V

AAGAGTGTGT AGGAAATGAT AGAGTGGAGC AGTATACAAG TAATGGGTTG   700
K  E  C  V    G  N  D    R  V  E    Q  Y  T  S    N  G  L

GTTGTGTGGA GTGGGAATGA TGATGAATGC CATGACTCAA GGTGGCATCA   750
 V  V  W    S  G  N  D    D  E  C    H  D  S    R  W  H  H

TGGTGCTACG GGAGTTATTT CTGTCACCAG CAACTTGGTT CCTGGTCTGA   800
  G  A  T    G  V  I    S  V  T  S    N  L  V    P  G  L

TGCGTGAACT AATGTTTGGA GGAAATAACC CTGTTTTGAA CTCAAAGCTA   850
M  R  E  L    M  F  G    G  N  N    P  V  L  N    S  K  L

ATGCCCTTGG TTGAATGGTT GTTTCATGAG CCAAACCCCA TTGCTCTGAA   900
 M  P  L    V  E  W  L    F  H  E    P  N  P    I  A  L  N

TACAGCTTTA GCTCAACTTG GGGTTGTGAG GCCTGTTTTT CGCCTTCCCT   950
  T  A  L    A  Q  L    G  V  V  R    P  V  F    R  L  P

ATGTTCCACT TACAAAGGCA AAGAGAGAGG AGTTTGTGAA GATTGTTGAG  1000
Y  V  P  L    T  K  A    K  R  E    E  F  V  K    I  V  E

AAAATTGGAC AGGAAAACTT TATTGGGGAA AGAGATGTCC AAGTTTTGGA  1050
 K  I  G    Q  E  N  F    I  G  E    R  D  V    Q  V  L  D

TGATATTGAT TTTTTTTTTG GTTGGTAGGT ATTAGTTTTT CCCCAATTGG  1100
  D  I  D    F  F  F    G  W

TATTGAGAAG TAAGTTTTTG TTGTTAGCGG CTTAATTTGA AAAATTTCTT  1150

GACATGAAGA TATCTTCTTT ATGTTGTTTT TTAAAAAAAA             1190
```

Fig. 2B

```
AGTCATTGGA AACACAGGAA GCTGTTCCAC GAGGGAAGCA ATCCATGCGA   440
 V  I  G    N  T  G    S  C  S  T    R  E  A    I  H  A
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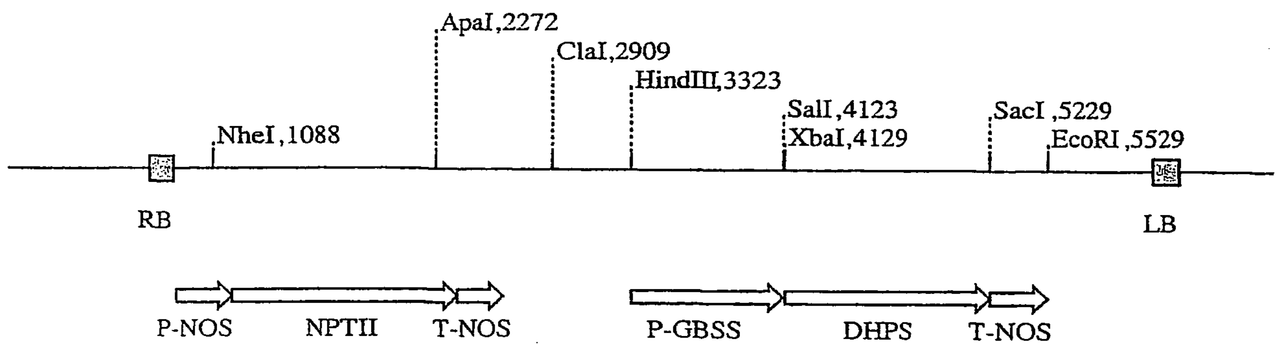
SUBSTITUTE SHEET (RULE 26)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ClaI,2909
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SalI,4123
XbaI,4129
SacI,5229
EcoRI,5529
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RB
LB
P-NOS
NPTII
T-NOS
P-GBSS
DHPS
T-NOS

Fig. 3

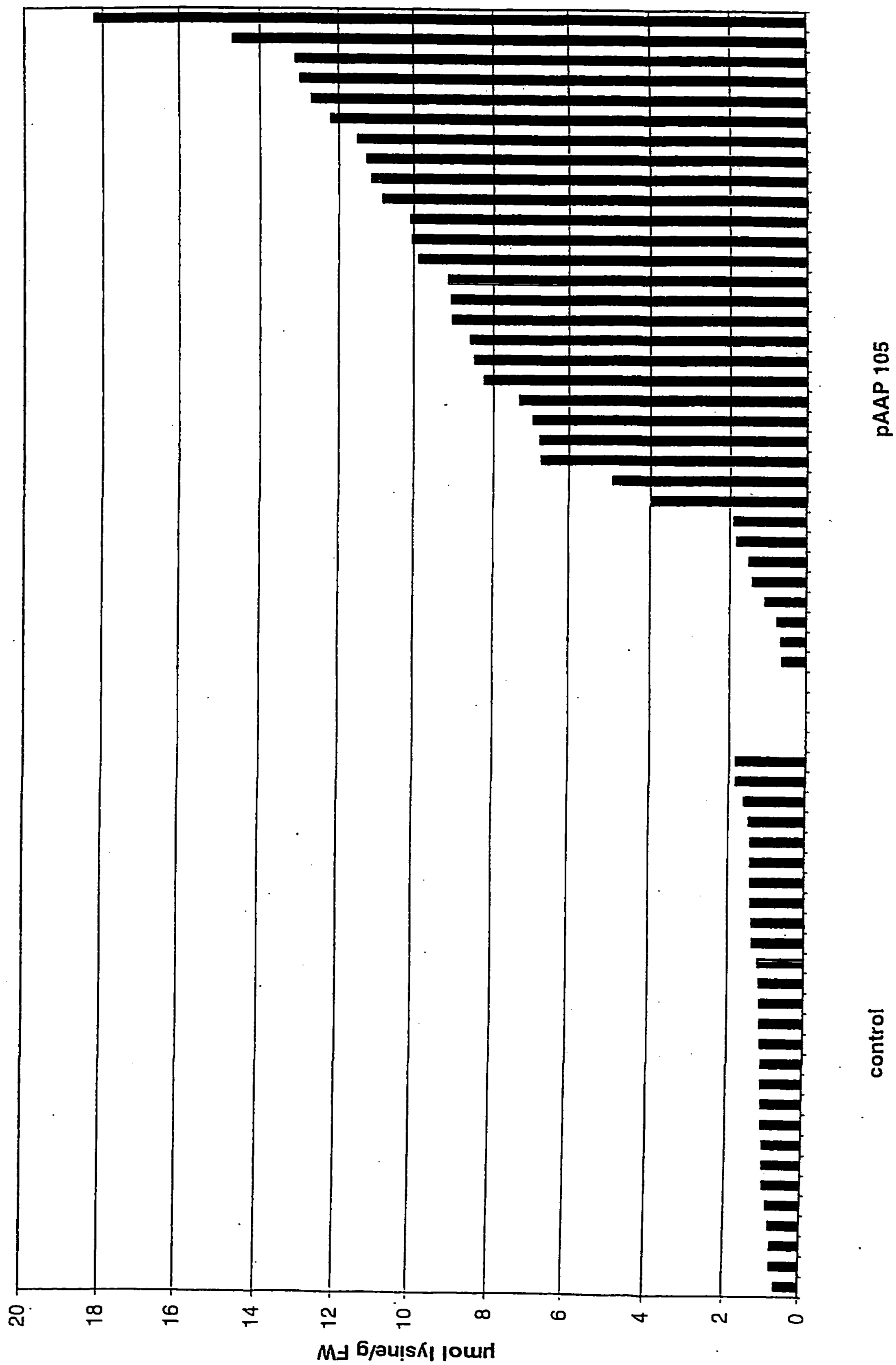


Fig. 4

Fig. 5

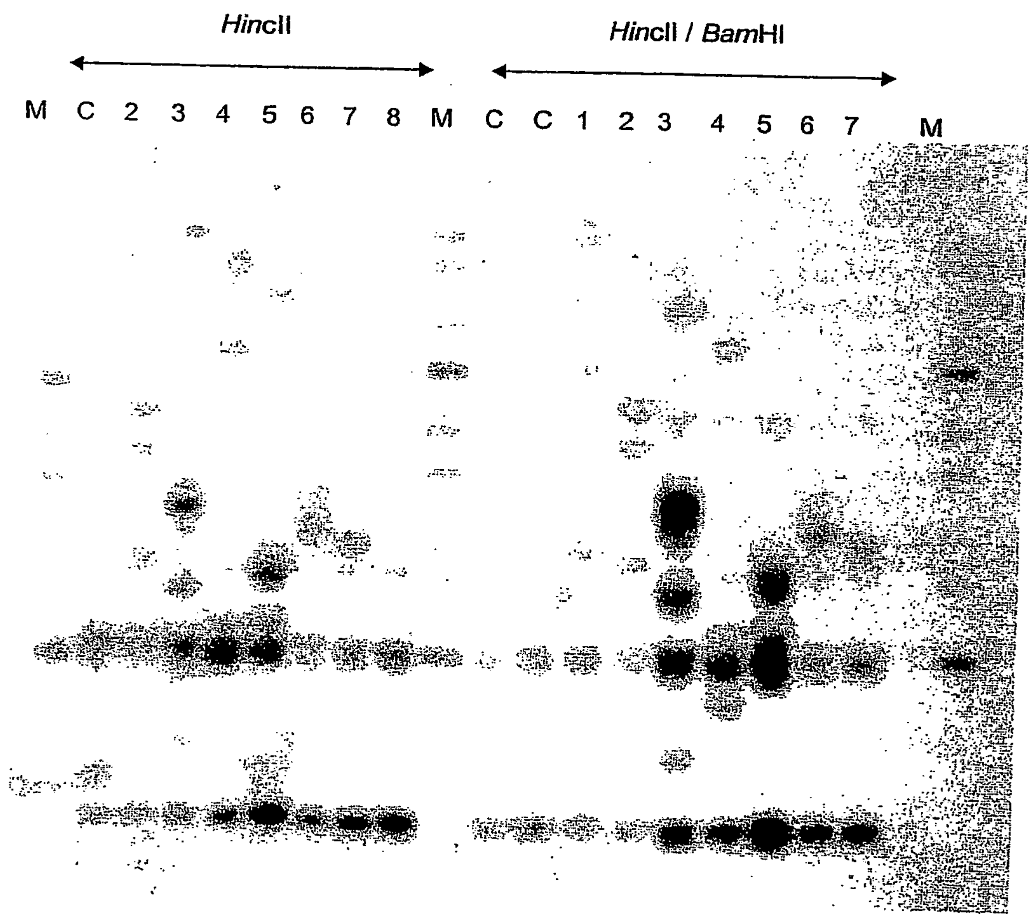
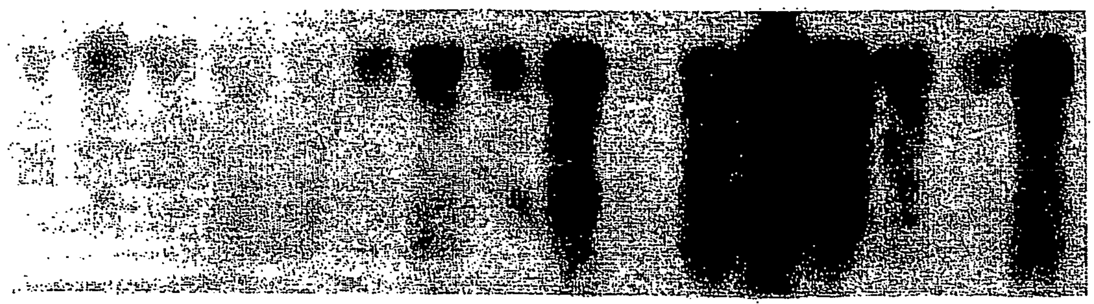


Fig. 6



INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL 00/00942

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/82 C12N9/88 C12N5/04 A23K1/16 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A23K A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, EPO-Internal, MEDLINE, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SHAVER JONATHAN M ET AL: "Single-amino acid substitutions eliminate lysine inhibition of maize dihydrodipicolinate synthase." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 93, no. 5, 1996, pages 1962-1966, XP000918975 1996 ISSN: 0027-8424 cited in the application page 1963, left-hand column, paragraph 2	15, 17-19, 22-26
A	paragraph ‘0002!; figure 2	1-6,9-23

-/--

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 July 2001	27.07.01

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Nichogiannopoulou, A

INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL 00/00942

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	GHISLAIN MARC ET AL: "A dinucleotide mutation in dihydrodipicolinate synthase of Nicotiana sylvestris leads to lysine overproduction." PLANT JOURNAL, vol. 8, no. 5, 1995, pages 733-743, XP000940840 ISSN: 0960-7412 cited in the application page 734, left-hand column, line 13	15, 17-19, 22-26
A	figures 4,6	1-6,9-23
A	BLICKLING STEFAN ET AL: "Feedback inhibition of dihydrodipicolinate synthase enzymes by L-lysine." BIOLOGICAL CHEMISTRY, vol. 378, no. 3-4, 1997, pages 207-210, XP000940920 ISSN: 1431-6730	1-26
Y	page 208, right-hand column, line 7; figure 3	7-26
Y	VAUTERIN MARC ET AL: "Isolation of a poplar and an Arabidopsis thaliana dihydrodipicolinate synthase cDNA clone." PLANT MOLECULAR BIOLOGY, vol. 25, no. 3, 1994, pages 545-550, XP000940934 ISSN: 0167-4412 cited in the application the whole document	7-26

International application No.
PCT/NL 00/00942

INTERNATIONAL SEARCH REPORT

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [ ] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. [X] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[X] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-6 all completely and 9-26 all partially

   Nucleic acid molecules, vectors, host cells, selection methods, plants and food or feed relating to mutated DHPS genes. Also a method to increase lysine content of food or feed by transformation with mutant DHPS genes.

2. Claims: 7 and 8 all completely and 9-26 all partially

   As invention 1 but relating to wild type potato DHPS